## Adenoviral gene therapy combined with (selective) chemotherapy for the treatment of cancer

Oosterhoff, D.

2005

# Adenoviral gene therapy combined with (selective) chemotherapy for the treatment of cancer

**Dinja Oosterhoff**

The research described in this thesis was performed within the framework of the Oncology Graduate School Amsterdam (OOA) at the Department of Medical Oncology (head: prof.dr G. Giaccone), VU University Medical Center, Amsterdam, The Netherlands. The research was financially supported by an educational grant from Aventis.

Publication of this thesis was financially supported by Ortho-Biotech, Pfizer B.V. and the Oosterhoff support group.

Cover: Staining of carboxylesterase-2 in colon cancer cells infected with the conditionally replicating adenovirus Ad.Δ24.E3-sCE2. Photo: G.L. Scheffer, R.R. Otsen.

Printed by Ponsen en Looijen, Wageningen

ISBN: 90-9019423-1

VRIJE UNIVERSITEIT

# Adenoviral gene therapy combined with (selective) chemotherapy for the treatment of cancer

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr.T.Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op vrijdag 17 juni 2005 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

**Dinja Oosterhoff**

geboren te ’s-Gravenhage

| | |
|---|---|
| promotor: | prof.dr. H.M. Pinedo |
| copromotoren: | dr. W.R. Gerritsen |
| | dr. V.W. van Beusechem |

*Der Blick des Forschers fand nicht selten mehr, als er zu finden wünschte.*

*Gotthold Ephraim Lessing (1729-1781)*

voor mijn ouders

**CONTENTS**

# Chapter 1

# General introduction

Partly published in Journal of Experimental Therapeutics
and Oncology 2004, 4: 37-57

# INTRODUCTION

Cancer is the second most common cause of death in the Western world. In the year 2000, in the Netherlands 69000 cases of cancer were diagnosed, of which 35500 in males and 33500 in females. Because 10 percent of the cases were diagnosed in patients already known to have some form of cancer, the number of new cancer patients approximated 62000. In the same year, 38000 patients died of cancer, which is more than 25% of the total number of deaths. The most common type of cancer was breast cancer, followed by colorectal cancer (13 percent of all cases in both sexes), lung cancer and prostate cancer. In this thesis, the main focus will be on colorectal cancer. Approximately 30% of all patients with colorectal cancer have metastatic disease at diagnosis, and 50% of early-stage patients will eventually develop metastatic or advanced disease.

The paucity of effective agents in the treatment of colorectal cancer in the past resulted in extensive investigation of 5-fluorouracil (5-FU) and 5-FU-based combinations. This agent has been developed in many different schedules of administration. Modulation of 5-FU anticancer effects with leucovorin became one of the standard treatment regimens for metastatic colorectal cancer. Additional pharmacological strategies to enhance the effectiveness of 5-FU included combination therapy with methotrexate, cisplatin, N-[phosphonacetyl)-L-aspartic acid (PALA) and interferon. Despite these attempts, no survival advantage was established until the development of the newer cytotoxic drugs CPT-11 and oxaliplatin.

Chemotherapy with cytotoxic drugs has demonstrated to be effective in prolonging survival and time to disease progression in patients with metastatic colorectal cancer [1]. Additionally, improvement in convenience of drug administration has been achieved with the development of oral fluoropyrimidines for the treatment of metastatic colorectal cancer. However, the success of chemotherapy is still limited by several drawbacks, including insufficient drug concentrations in the tumor, systemic toxicity, lack of selectivity for tumor cells over normal cells, and the appearance of drug-resistant tumor cells. A number of strategies have been used to overcome these problems, including alternative formulations, resistance modulation, toxicity modifiers and gene therapy.

Gene therapy involves the insertion of a gene into somatic cells in a way that sufficient quantities of the therapeutic gene will be expressed. The basic concept of human gene therapy has a history of more than 30 years [2]. Since the early days of recombinant DNA technology, the introduction of foreign DNA for therapeutic intervention has been a major goal and this has led to the development of a variety of gene therapy strategies.

One promising area for improving tumor selectivity using an gene therapy strategy is Gene-Directed Enzyme Prodrug Therapy (GDEPT). GDEPT, also known as suicide gene therapy, is a two-step approach. In the first step, a gene encoding a drug-activating enzyme is expressed specifically in tumor cells. In the second step, a non-toxic prodrug, a substrate of the enzyme that is now expressed in the tumors, is administered to the patient. The net gain is that a systemically administered prodrug can be converted to high local concentrations of an active anticancer drug in the tumor, resulting in increased efficacy of the chemotherapeutic drug while decreasing the side effects of the drug. To be successful, both enzymes and prodrugs should meet certain requirements for this strategy. The enzyme should be a protein that is only expressed in low concentrations in human tissues so that without GDEPT the majority of prodrug is not converted into the toxic drug. The protein must achieve sufficient expression in the tumors and have high catalytic activity. The prodrug should be a good substrate for the expressed enzyme in tumors, but should not be activated by endogenous enzymes in normal human tissues. In

case the prodrug-converting enzyme is an intracellular enzyme, the prodrug should be able to cross the tumor cell membrane for intracellular activation, and the cytotoxicity differential between the prodrug and its corresponding activated drug should be as high as possible.

The disadvantage of this approach is that the prodrug-converting enzyme will not be localized throughout a solid tumor mass. Up to date, the transduction efficiency of all available vector systems is inadequate. Vector tropism and the high pressure and limited blood supply in the central region of a tumor hamper efficient transduction of all tumor cells. Therefore, clinically successful GDEPT relies heavily on the so-called bystander effect, which will increase the anti-tumor effect. The bystander effect, initially described by Moolten *et al*. [3] can be defined as an extension of the killing effects of the active drug to untransfected, neighboring cells. This implies that even if only a small percentage of the target cells are genetically modified and express the therapeutic gene, tumor eradication may still be achieved. The bystander effect is crucial for a successful GDEPT strategy, since with the protocols currently adopted in clinical trials, the transfection efficiency is unlikely to be greater than 10%. A bystander effect can be achieved via two ways. First of all, it might be achieved by diffusion of the active drug to adjacent non-expressing tumor cells. Secondly, one can increase the bystander effect by secretion of the prodrug converting enzyme from transduced tumor cells, so that the enzyme can penetrate through a solid tumor mass. If hereafter the prodrug is administered, it will be activated extracellularly throughout the tumor, leading to toxicity to untransduced neighboring cells. To prevent leakage of the secreted enzyme into the circulation that could lead to unwanted side effects, the secreted enzyme could be targeted to tumor antigens (Figure 1). Evidence in animal models suggests that a systemic immune response may also play an important role in inducing bystander killing [4]. The presence of an intense inflammatory infiltrate has been described in regressing tumors of immunocompetent animals treated with GDEPT systems [5-9].

In this Introduction chapter, replication deficient as well as conditionally replication competent adenoviral vectors as vehicles to deliver therapeutic genes to tumor cells will be discussed. Furthermore, GDEPT with different enzyme prodrug models will be discussed, where the focus is on the use of GDEPT with the enzyme carboxylesterase (CE) for tumor specific conversion of the prodrug CPT-11 for the treatment of colorectal cancer.

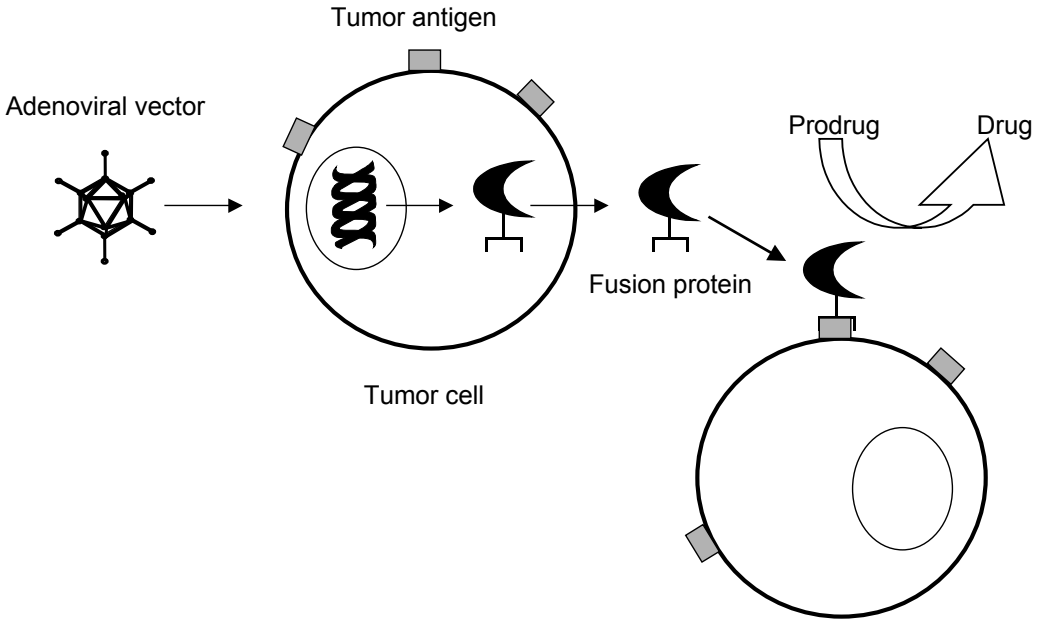



***Figure 1:*** *Schematic presentation of GDEPT with a gene encoding a secreted, tumor targeted enzyme. This gene is cloned into an adenoviral vector that is used to infect tumor cells. The infected cells will express and secrete the fusion protein. The secreted fusion protein will diffuse through the tumor and will bind to antigen expressing neighboring cells. If hereafter the non-toxic prodrug is administered, it will be converted to the active drug extracellularly, after which the generated drug can further diffuse through a tumor and kill the tumor cells.*

## GENE-DIRECTED ENZYME PRODRUG THERAPY (GDEPT)

### *GDEPT with thymidine kinase to activate ganciclovir*

Many GDEPT systems have been described in the past decade. The most extensively studied enzyme prodrug models are the herpes simplex virus thymidine kinase (TK)/Ganciclovir (GCV) and the cytosine deaminase (CD)/5-fluorocytosine (5-FC) combinations. GCV is an antiviral drug that is phosphorylated by TK and then by cellular kinases to produce GCV triphosphate, which disrupts DNA synthesis during S-phase, leading to cell death. In the last 15 years, more than 600 papers have discussed the potentiality of TK/GCV for cancer gene therapy. Preclinical studies using adeno- and retroviral vectors were performed in many different animal models and successful results were reported for established rodent liver metastases [5], murine hepatocellular carcinomas [9], rodent glioblastomas [10], human head and neck carcinomas [11], human mesotheliomas [12] and several other tumor types. One of the main drawbacks of this enzyme prodrug model is that the highly charged triphosphate is insoluble in lipid membranes. This impairs the diffusion of the drug and makes cell-to-cell contacts necessary for bystander killing. Nevertheless, preclinical studies showed that tumor regression could be achieved when only 10% of the tumor cells expressed *TK* [5,13]. This phenomenon has been proposed to result from transfer of activated GCV through gap junctions [14-17] or exchange of apoptotic vehicles [13,18]. It is likely that a major part of the *in vivo* bystander killing is mediated by the host immune system. TK/GCV treatment resulted in infiltration of CD4+ and CD8+ T-cells and macrophages as well as increased expression of cytokines [6,19]. An immune-related anti-tumor response could also account for the 'distant bystander effect'. GCV treatment of head and neck squamous cell carcinoma xenografts in nude mice resulted not only in the eradication of *TK* expressing tumors, but also in delayed regression of untransduced tumors in the contra-lateral flanks [20], which was abrogated in SCID mice.

On the basis of many animal studies, the first gene therapy trials using TK/GCV to treat ovarian cancer was approved in 1991, and since then several other clinical studies have been undertaken. An overview of clinical trials performed with GDEPT is shown in table 1. With TK and GCV, gene therapy trials of brain tumors [21-27], metastatic melanoma [28] and prostate carcinoma [29,30] have been performed. Delivery of the gene has been done by injecting *TK*-containing replication deficient adenoviruses or retroviral vector-producing cells. In these phase I clinical trials, only moderate toxic events were reported, which were mostly resolved at the termination of the therapy. Moderate therapeutic responses were observed in some of the patients. In a phase I/II study for recurrent glioblastoma, injection of retroviral producing cells in the surgical cavity margins after tumor debulking followed by intravenous GCV resulted in the absence of recurrence in four of 12 patients at four months and in one patient at 2.8 years after treatment [26]. Relatively poor responses could be due to insufficient gene transfer and limited distribution within the tumor mass.

There are numerous ways of ameliorating treatment efficacy, notably through the improvement of gene delivery and a better understanding of the molecular mechanism of the bystander effect. Significant benefits could also arise from the introduction of new nucleoside analogues with a higher affinity for TK and fewer side effects than GCV [31-34] and of TK mutants engineered to increase specificity and activity towards the prodrug [35,36].

***GDEPT using cytosine deaminase to activate 5-FC***

The system consisting of CD and 5-FC is based on the production of a toxic nucleotide analogue. The enzyme CD is found in certain bacteria and fungi, but not in mammalian cells, and catalyzes the hydrolytic deamination of cytosine to uracil. It can therefore convert the non-toxic prodrug 5-FC into 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to potent pyrimidine antimetabolites. 5-FU is widely used in cancer chemotherapy and is a drug often given to patients with colorectal cancer. Administration of 5-FU causes a lot of side effects and high dose levels are required for tumor response. The *CD* gene used for GDEPT has been cloned from *Escherichia coli* [37] and has been shown in a number of *in vitro* studies to enhance mammalian cell sensitivity to 5-FC up to 2000 fold [37,38]. *In vivo* anti-tumor activity has been demonstrated in several animal models, including fibrosarcomas [39], carcinomas [40-43], gliomas [44] and metastatic lesions of different origin [7,45]. One of the main advantages of CD/5-FC enzyme prodrug therapy is that no cell-to-cell contact is required for the bystander effect, since 5-FU can diffuse in and out cells by non-facilitated diffusion. Experiments conducted *in vitro* demonstrated that 1-30% of cells expressing *CD* could generate sufficient 5-FU to completely inhibit growth of all cells [41,44].

A phase I trial involving local injection of a plasmid containing the *CD* gene, regulated by the tumor selective erbB-2 promoter and systemic 5-FC administration in breast cancer patients demonstrated the safety of this approach [46]. In 11 of 12 patients *CD* expression was demonstrated, whereas in 4 patients tumor regression was observed, whereas two of them did not even receive the prodrug.

***Table 1:*** *Clinical trials with GDEPT*

| Prodrug system | Tumor type | Phase | Description |
|---|---|---|---|
| TK/GCV | Malignant glioma | I | replication deficient adenovirus, intratumoral injection [22,47] |
| | Malignant brain tumors | I-II | retroviral vector producer cells, intracerebral injection [21,23-27,48] |
| | Malignant glioma | III | replication deficient retrovirus, intratumoral injection [49] |
| | Prostate cancer | I | replication deficient adenovirus, intraprostatic injection [29,50] |
| | Prostate cancer | I-II | replication deficient adenovirus, combined with radiotherapy and hormonal therapy [51,52] |
| | Hepatic metastases from colorectal cancer | I | replication deficient adenovirus, intratumoral injection [53] |
| | Metastatic melanoma | I-II | retroviral vector producer cells, injected in tumor nodules [28] |
| | Mesothelioma | I | replication deficient adenovirus, intratumoral injection, combined with corticosteroids [54,55] |
| CD/5-FC | Breast cancer | I | plasmid construct, CD driven by the Erb-2 promoter, intratumoral injection [46] |
| | Hepatic metastases from colorectal cancer | I | replication deficient adenovirus, intratumoral injection [56] |
| CD/5-FC +TK/GCV | Prostate cancer | I | replication competent adenovirus, intraprostatic injection [57] |
| Ntr/CB1954 | Liver tumor or colon metastases in liver | I | replication deficient adenovirus, intratumoral injection [58] |

Specific gene expression and prodrug activation offers the possibility of combining Gene-Directed Enzyme Prodrug Strategy systems to enhance the antitumor activity of the single treatments without increasing systemic toxicity. Delivery of the CD-HSV-TK fusion gene followed by GCV and 5-FC treatment sensitized gliosarcoma, mammary carcinoma and prostate tumor cells to the prodrugs [59-62]. The combination of these two suicide genes has also been shown to sensitize the tumor cells to irradiation [61,63].

***Enzyme prodrug therapy with CE and CPT-11***

A last enzyme prodrug model that will be discussed is the use of the enzyme CE to activate the prodrug CPT-11. The prodrug and the prodrug-converting enzyme will each be discussed in more detail in the following sections.

***CPT-11***

CPT-11 (irinotecan or 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) is a water-soluble semi-synthetic derivative of the natural alkaloid camptothecin, a relatively new anti-neoplastic agent. Camptothecin was originally isolated from the Chinese/Tibetan ornamental tree *Camptotheca acuminata*, commonly known as the 'Chinese tree of joy'. CPT-11, of which the molecular structure is shown in figure 2, was first discovered and synthesized in Japan in 1983 and is a chemotherapeutic agent that causes S-phase-specific cell killing by inhibition of topoisomerase I (topo I) in the cell. Topoisomerase I relaxes supercoils of DNA arising during DNA replication and transcription and repair recombination [64]. The mechanism of action of topo I can be divided into several steps. First, the enzyme binds to the double stranded DNA. Subsequently, the enzyme cleaves and reseals the phosphodiester backbone of DNA, which allows passage of another singe-or double stranded DNA through the nicked DNA. Finally, the cleaved DNA strand is ligated for subsequent replication or transcription. CPT-11 act by binding non-covalently to the DNA-Topo I cleavable complex and interferes with DNA religation. Probably, CPT-11 interacts with both the enzyme and the DNA, resulting in stabilization of the cleavable complex and accumulation of single-strand breaks in the DNA. These single strand breaks are by themselves not sufficient to cause cell death. However, upon their collisions with the advancing replication forks, the formation of a double-strand DNA break occurs, leading to irreversible arrest of the replication fork and cell death [65]. The collision of the complex with the replication fork also results in G2 arrest/delay by signaling the presence of DNA damage to an S-phase checkpoint mechanism [66]. At higher concentrations of CPT-11, non S-phase cells can also be killed. The mechanism of non-S-phase cell killing appears to be related to transcriptionally mediated DNA damage and through apoptosis [67].

CPT-11 has demonstrated anti-tumor activity in immune deprived animals bearing human tumor xenografts and is approved for use in the treatment of metastatic colorectal cancer in humans [68-74]. Furthermore, CPT-11 is currently being tested for its efficacy in a wide range of tumors, including non-small-cell lung cancer, rhabdomyosarcoma and neuroblastoma. Initial approval in the United States was as second-line treatment for metastatic colorectal cancer [75] and more recently it has been approved for use in combination with 5-FU/Leucovorin as a first-line treatment for this disease [76,77]. Likewise, phase II studies on advanced esophageal and gastric cancer showed encouragingly high response rates [78]. The major toxicities of CPT-11 in clinical use are myelosuppression and

diarrhea. The drug can cause either acute diarrhea or a delayed diarrhea syndrome, which is possibly related to the accumulation of the active metabolite of CPT-11 in the bowel [79].

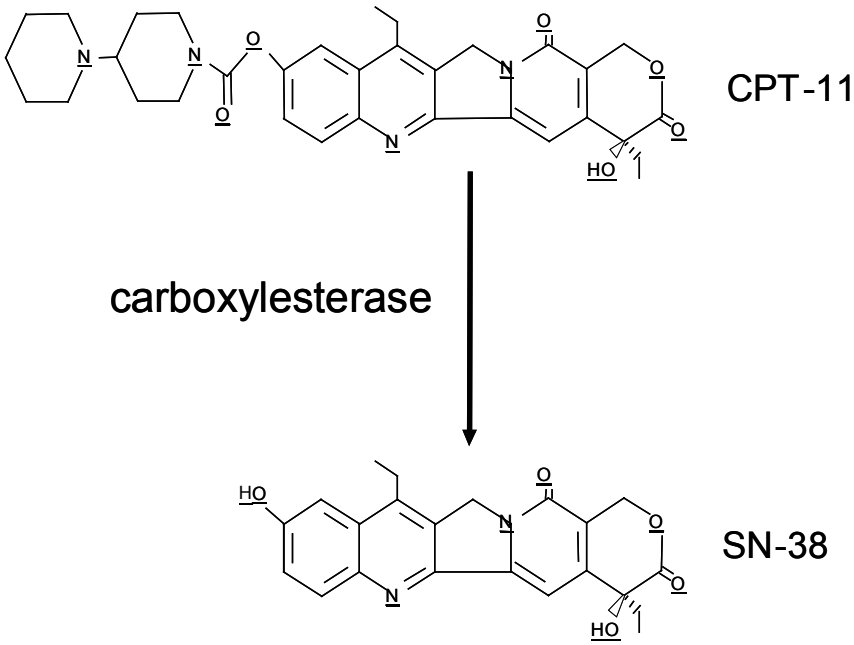



***Figure 2:*** *Molecular structure of the prodrug CPT-11 that can be converted into the active drug SN-38 by the enzyme carboxylesterase*

CPT-11 is a prodrug since it needs to be activated to the drug SN-38 (7-ethyl-10-hydroxycamptothecin) by cleavage of the bulky dipiperidino side chain at the carbon position [80,81] and this conversion mainly takes place in the liver and the small intestine. CPT-11 undergoes extensive hepatic metabolism as shown in figure 3. Two major human liver CEs, CE1 and CE2 [82] can hydrolyze CPT-11 to generate the active drug SN-38. Oxidative metabolism of CPT-11 by cytochrome P450 isoenzymes results in formation of two major metabolites, APC (7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin) and NPC (7-ethyl-10-[4-(1-piperidino)-1-amino] carbonyloxycamptothecin) [83,84]. *In vitro* studies have demonstrated that among the cytochrome P450 enzymes, only CYP3A4 can oxidize CPT-11 to APC or NPC [85]. SN-38 is inactivated by glucuronidation to form SN-38 glucuronide (SN-38G). Several uridine diphosphate glucuronysyltransferase (UGT) isoforms were studied, and UGT1A1 was found to be at least 10 times more active than other isoforms [86]. *In vitro* studies demonstrate that NPC as well as APC can be metabolized by CE to produce SN-38 [83,87]. Butyrylcholinesterases can also convert CPT-11 to SN-38, although the exact contribution that these enzymes play in drug metabolism remains unclear [88].

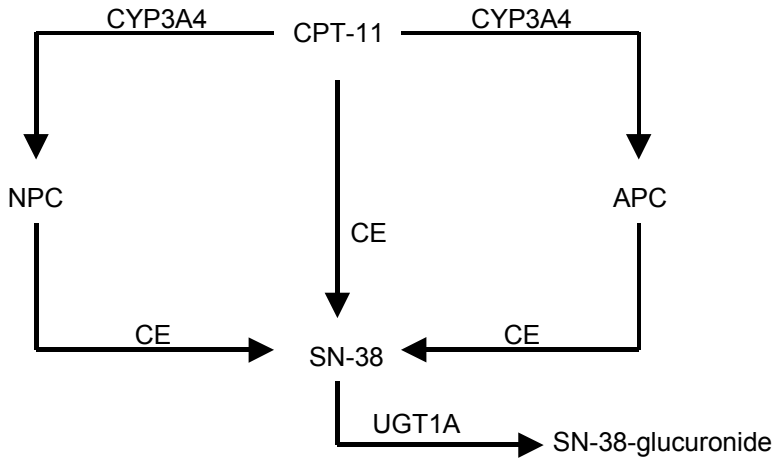



***Figure 3:*** *Schematic presentation of the metabolism of CPT-11. CPT-11 is oxidized by CYP3A4 to form APC and NPC. NPC, APC and CPT-11 are metabolized by CEs to form SN-38. SN-38 is inactivated by UGT1A to SN-38 glucuronide.*

Following intravenous administration of CPT-11 to humans, low concentrations of SN-38 are observed in the plasma of patients [89], ranging from 1% to about 5% prodrug converted to the active drug for most schedules of administration. One study further demonstrated that the percentage of CPT-11 converted to SN-38 decreased as the drug dose increased, indicating limited availability of the enzyme responsible for this conversion. Thus, increasing the dose of CPT-11 does not lead to an additional therapeutic benefit [90]. In light of the curative potential of CPT-11 when sufficient plasma levels can be reached [72], the potential for tumor specific expression of active *CEs* to increase SN-38 levels in tumor cells and dramatically increase the therapeutic index merits further investigation.

***Carboxylesterases***

CEs belong to a large family of serine-active hydrolases with a 60 kDa subunit mass and have a characteristic β-α-β esterase fold and catalytic acid. Humans express *CEs* in the liver [91,92], plasma [93], small intestine [94], brain [92,95], stomach [95,96], colon [95], macrophages [97] and monocytes [98]. CEs are classified in four major groups by amino acid sequence alignment [99], CES1, CES2, CES3 and CES4, and several subgroups. In general, CEs exhibit about 80% sequence identity within a CES group. All the isoenzymes are responsible for the hydrolysis of many exogenous compounds, the consequences of which include both inactivation of drugs and activation of prodrugs. Human liver CE activates the prodrugs CPT-11 and lovostatin [100] and conversion of a prodrug of prostaglandin F2α has been reported [101]. A significant number of drugs and endogenous compounds are substrates of CEs, including dipivefrin hydrochloride [102], carbonates [103], cocaine [104], haloperidol [105] and steroids [95]. The most abundant and well-studied human enzymes are CE1 (GI: 119576) and CE2 (GI: 4504565) that belong to classes CES1 and CES2 respectively. The mRNA of both isoforms is highly expressed in liver. Both *CE1* and *CE2* are also expressed in colon, heart, intestine, testis and kidney tissue. The implications for drug metabolism are that both enzymes are important for systemic clearance of esters from blood through the liver. Since *CE1* is more abundantly expressed in the kidneys it seems to have a more pronounced role than CE2 in clearance via the kidney, whereas CE2 is more important than CE1 for clearance of orally administered drugs through the small intestine and colon.

Although substrate specificity of these isoenzymes is overlapping, they do show substrate preference [106]. Human CE1 prefers substrates with a smaller alcohol moiety and larger acyl substitutes such as meperidine or methylphenidate, whereas CE2 prefers large alcohol and small acyl moieties such as CPT-11 or heroin. The N-terminus of nascent CE2 contains a 19-residue signal peptide that directs the protein to the endoplasmatic reticulum [107]. These amino acids are, however, not present in the mature protein. The four C-terminal amino acids HXEL, of which the X can be any amino acid, anchor the protein within the endoplasmatic reticulum. Deletion of these amino acids results in secretion of the enzyme from the cell. The secreted enzyme is functional, catalyzing the metabolism of both simple and complex CE substrates [108].

***GDEPT with CE/CPT-11***

Three different CEs have been studied in the context of selective activation of CPT-11, i.e. human liver CE1 and CE2 and rabbit CE (rCE). In context of conversion of CPT-11, CE2 has a 64-fold higher catalytic efficiency than CE1 [82]. This was unexpected, because until today rCE is the most efficient CPT-11 converting enzyme, and CE1 demonstrated to have greater than 81% similarity to rabbit CE, whereas CE2 only shares about 40% homology. In *in vitro* assays, the IC50 values for CPT-11 in human tumor cell lines expressing *rCE* were 8-80 fold lower than for plasmid-transfected control cells [109-112]. Similar to the results obtained with cell lines, stably *rCE* expressing human tumor xenografts were sensitized to CPT-11, since complete, long-term regression was observed following administration of CPT-11 to xenograft bearing mice [112].

Several studies have been performed using CPT-11 in combination with different CEs in a GDEPT approach. Kojima *et al*. described the construction of a replication deficient adenoviral vector containing the human liver *CE1* gene driven by the CMV promoter [113,114]. *In vitro* results showed that several tumor cell lines infected with this virus express *CE1* and in the presence of CPT-11 tumor growth was effectively suppressed. However, on many other tumor cell lines only minimal effects were observed. This underscored the notice that the success of a GDEPT approach for CPT-11 requires an enzyme with a high efficiency of converting CPT-11 to SN-38. rCE was found to be 100-1000 fold more efficient in converting CPT-11 than human liver CE1 and was 12-55 fold more efficient in sensitizing transfected cells to CPT-11 [112]. Therefore, an adenoviral vector expressing *rCE* was constructed and transduction of human tumor cells led to sensitization to CPT-11 [115]. This virus, Ad-rCE, has been used for the selective eradication, or purging, of neuroblastoma cells from bone marrow or peripheral stem cells autologous stem cell rescue. Ad-rCE showed to selectively transduce tumor cells in mixtures of hematopoietic/neuroblastoma cells. Administration of CPT-11 subsequently, resulted in cytotoxicity specifically to neuroblastoma cells. For GDEPT applications of solid tumors, however, the disadvantage of rCE is that expression of a nonhuman protein in patients may lead to an immunological response and subsequent enzyme inactivation. A human enzyme with higher affinity and higher efficiency than CE1 may overcome these limitations. Since it was shown that human CE2 has a higher affinity and a higher conversion velocity for CPT-11 than CE1 [82], in this thesis we investigated if CE2 could be a candidate to employ in a GDEPT approach to treat human colon caner tumors.

As stated previously, to achieve efficient kill of all tumor cells, a bystander effect is required, whereby CPT-11 is cleaved to SN-38 that not only kills the tumor cells in which CE is formed, but also neighboring tumor cells that do not express *CE*. Although SN-38 is able to freely pass the cell membrane and might thus exert cytotoxic effects on

neighboring cells, we hypothesized that extracellular conversion of CPT-11 would lead to an increased bystander effect compared to intracellular prodrug conversion. A disadvantage of a secreted enzyme, however, might be that the enzyme is capable of leaking away from the transduced tumor cells into the circulation, resulting in conversion of prodrug outside of the tumor area and thus negatively affecting tumor selectivity of GDEPT. A way to prevent leakage of the enzyme from the tumor may be secretion of a fusion protein consisting of an scFv antibody and CE by transduced tumor cells. Such a fusion protein will bind specifically to tumor cells, thereby preventing leakage into the circulation and reducing the chance that side effects occur.

## A TARGET MOLECULE FOR THE FUSION PROTEIN: EPITHELIAL CELL ADHESION MOLECULE

In order to achieve the highest specificity of enzyme prodrug therapy one could target the prodrug-converting enzyme specifically to tumor cells by constructing fusion proteins consisting of the enzyme and the binding part of an antibody. Like the prodrug converting enzyme, the target antigen and the targeting antibody must meet certain requirements. First, the target antigen should be expressed preferentially on tumor cells. In the most optimal situation, healthy cells do not express the target antigen whereas it is highly expressed in tumor cells. The antigen should be localized on the tumor cell surface or should be secreted into the extracellular matrix of the tumor, but no shedding in the blood should occur. An example of an interesting tumor antigen meeting these requirements is the Epithelial Cell Adhesion Molecule (EpCAM).

EpCAM has first been identified as a tumor-specific antigen on several carcinomas of different origin. Several independent studies generated different antibodies against this tumor specific molecule expressed on carcinomas. Because the c-DNA has been independently cloned by a number of groups [116-119], the molecule is known by many different names, i.e., the human pan-antigen epithelial glycoprotein EGP40, CO-171A antigen, KSA1/4, ESA, GA733-2, MOC31 and so forth. In the early 1990s, the reports on the carcinoma antigens were combined and it became clear that the described molecules were virtually identical.

EpCAM is a type I transmembrane glycoprotein and consists of an extracellular domain containing two epidermal growth factor-like repeats and a short intracellular domain of 26 amino acids in which 2 binding sites for $\alpha$-actinin are present for linkage to the cytoskeleton [120]. It is a relatively small protein that is highly conserved during evolution and mediates calcium-independent homotypic cell-cell adhesions. Studies in murine fibroblasts transfected with *EpCAM* revealed that the molecule is associated with proliferation [121,122]. Upon (over)expression of *EpCAM*, cadherin associations dissociate, which leads to accumulation of detergent soluble E-cadherin/$\beta$-catenin complexes, and to a decrease in total cellular $\alpha$-catenin [123]. This suggests that during cell division, the strong tight E-cadherin mediated cellular adhesion is abrogated, while the weaker intercellular adhesion mediated by EpCAM still holds the cell in place [124]. After the proliferative phase, *EpCAM* expression declines and higher levels of E-cadherin mediate intercellular adhesions and direct cellular differentiation.

*EpCAM* is normally expressed at low levels at the basolateral membrane of the majority of epithelial tissues, except in adult squamous epithelium and some specific epithelial cell types, such as hepatocytes. Overexpression of *EpCAM*, as well as *de novo* expression was observed in colon carcinoma and in squamous carcinoma of the uterine

cervix [125,126]. Immunohistochemical staining of dysplastic colon cells showed overexpression of *EpCAM* not only on the basolateral membrane, but apical staining was observed as well. The association of *EpCAM* expression with metastases is less clear. One would expect to find higher *EpCAM* expression in metastasized cells, because these cells are more likely to escape the epithelium than well-differentiated cells anchored by E-cadherin mediated junctions. Momburg *et al*. demonstrated that micrometastasis originating from carcinomas could be detected with EpCAM antibodies [127]. However, in nodal metastasis originating from head and neck squamous carcinomas, *EpCAM* expression was found to be reduced compared to the primary tumor [128]. In contrast, Chaubal *et al*. concluded that *EpCAM* gene expression could be used as a tool to identify disseminated tumor cells [129,130].

This overexpression offers possibilities to target EpCAM for cancer immunotherapy or adenoviral gene therapy. Colorectal cancer has been targeted with the monoclonal antibody CO17-1A and anti-idiotypic antibodies mimicking the CO17-1A epitope. An improved survival was accompanied by a prolonged systemic immune reaction to the antibody [131]. Presently, its anti-tumor effect is being studied as monotherapy after resection of stage II colon cancer, and in combination with chemotherapy in patients with stage II or III rectal cancer [132]. Patients with resected Dukes' C colorectal carcinoma were treated with monotherapy in an adjuvant setting with edrecolomab, the murine monoclonal antibody that binds with low affinity to EpCAM [133]. This study showed that the antibody administered after surgery prevented the development of distant metastasis in approximately one-third of patients. The therapeutic effect was maintained after 7 years of follow-up.

EpCAM antibodies have also been used to target adenoviral vectors specifically to tumor cells. By construction of a chemical conjugate or a bispecific scFv antibody that is on one side directed to the adenoviral fiber and on the other side to the target antigen EpCAM, adenoviruses were retargeted to cancer cells expressing *EpCAM* [134,135]. As well on gastric cancer cell lines as on primary gastric cancer cells an improved ratio of tumor transduction over normal epithelium transduction was accomplished by the EpCAM targeted vectors [136]. All these studies demonstrate that EpCAM is a very interesting protein to target newly developed anti-cancer strategies specifically to tumor cells.

## ADENOVIRUSES

Adenoviruses (Ads), belonging to the family Adenoviridae, were first cultured and reported as unique viral agents in 1953 [137]. Ads are associated with the common cold and they cause respiratory, intestinal and eye infections in humans. There are more than 50 distinct human Ad serotypes and of these, types 2 and 5 have been extensively characterized and have served as valuable tools for the study of the molecular biology of DNA replication, transcription, and protein synthesis in mammalian cells.

The Ad particle is non-enveloped and is a regular icosahedron with a diameter of 70-90 nm. The capsid is composed of 252 capsomeres, with 240 forming the 20 triangular facets and 12 forming the 12 vertices. Each capsomere present on the facet is surrounded by 6 neighbors and is hence called a hexon (Figure 4) The 12 vertex capsomeres have only 5 neighbors and are called pentons [138]. Protruding from each penton is a structure called fiber, whose length varies with the serotype. At its distal end, the fiber bulges out to form a globular knob domain, which mediates the attachment of the virion to the cellular receptor.

The Ad is a DNA virus with a genome consisting of a linear double-stranded DNA molecule of approximately 36000 nucleotides and the entire genome sequences are known ([139], GenBank accession number BK000408). The genome has a virus-coded terminal protein at each 5' end of the linear genome [140] and inverted terminal repeats. The genome is functionally divided into two major overlapping regions, early and late, based on time of transcription after infection. The region *E1* is active immediately upon entry of the viral genome into the nucleus and it encodes proteins that regulate all of the other early functions [141,142]. The *E2* region encodes proteins involved directly in adenoviral DNA replication, whereas the *E3* region encodes proteins involved in reducing the antiviral immune response [143] and in effective lysis of the cell after viral replication has completed [144,145]. The *E4* region encodes proteins with multiple functions such as control of viral replication, DNA replication and shut-off of host protein synthesis, The late genes, initiated 8-h post infection, encode most of the viral structural proteins, including capsid proteins, hexon, penton and fiber, all transcribed from the major late promoter.

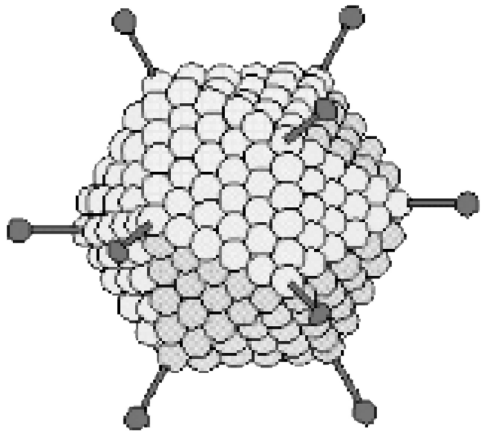


***Figure 4:*** *A schematic representation of the adenovirus showing the major proteins of the capsid: hexon, fiber and penton.*

Ad5 enters the cell by attachment of its fibers to the 46-kDa specific cell surface receptor coxsackievirus and Ad receptor (CAR) [146]. Once bound to the cell surface, the virus is internalized via receptor-mediated endocytosis. Receptors that facilitate internalization include the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. It then migrates within the plasma membrane to clathrin-coated pits that form endosomes. After release of the virion, it is transported to the nucleus via nuclear targeting signals where the various transcriptional regions are expressed.

Ads have been widely used to express foreign proteins and to transfer and express therapeutic genes. They have a number of advantages in that they have a broad host range and can infect proliferating and quiescent cells. Ad genomes do not integrate into the host genome, making them safe vectors for mediating transient transgene expression. Ad vectors can easily be purified to high titers. With mounting interest in gene therapy, many types of Ad vectors have been developed. Most studies performed to date have used the first generation (*E1* and *E3* deleted) or second-generation (*E1*, *E3* and *E4* or *E2* deleted) type 5 Ad vector. The use of replication deficient Ad vectors as gene delivery vehicles has reached clinical trials.

## CONDITIONALLY REPLICATING ADENOVIRUSES (CRAds)

Replication-defective Ad gene delivery vectors have so far shown modest anti-tumor efficacy because of poor transduction and penetration capacity in solid tumor masses. To overcome this limitation, one could use transgenes encoding secreted proteins, but also conditionally replicating adenoviruses (CRAds) were developed and explored as novel anti-cancer agents. CRAds replicate only in cancer cells and destroy these cells through the natural process of adenoviral replication. In addition, the generated progeny viruses released from infected and lysed cancer cells may infect neighboring tumor cells. Via several rounds of replication and cell lysis, the tumor will ultimately be destroyed. The selective replication of CRAds in cancer cells, the prevention of replication in normal cells, the efficacy of CRAds in clinical trials and various ways to increase CRAd efficacy will be discussed.

### *Mechanisms of selective replication of CRAds in cancer cells*

The replication cycle of adenoviruses consists of an early and a late phase, separated by the onset of DNA replication. A major function of the adenoviral early genes is to provoke the infected cell to enter cell cycle and progress to S-phase. In the S-phase the virus can take advantage of the cellular DNA replication machinery to replicate its own genome efficiently. For the induction of cell cycle progression, inactivation of the cellular retinoblastoma (pRb) and p53 tumor suppressor proteins is required (illustrated in figure 5A). These actions are brought about by proteins encoded by the Ad *E1* region. The *E1A* gene is the first to be transcribed and encodes proteins that bind members of the cellular pRb family [147]. This interaction results in release of the pRb bound transcription factor E2F, which is then free to activate transcription of E2F-responsive genes, involved in stimulating cell cycle progression.

To circumvent premature cell death during viral replication, the viral E1B-55kD protein binds to and inhibits p53, while the E1B-19kD protein functions as a viral homologue of the anti-apoptotic factor bcl-2 [148-150]. The remaining early regions encode proteins involved in viral DNA synthesis (*E2*), modulation of host immune response and cell lysis (*E3*), and regulation of viral gene expression, mRNA transport, DNA replication and apoptosis (*E4*). The late gene products encode viral structural proteins and proteins involved in virion assembly.

CRAds are made by modifying the Ad genome such that essential early functions are abrogated in non-malignant cells but not in cancer cells, or by deleting early viral functions that are essential for replication in non-malignant cells and are redundant in cancer cells.

### ***Limiting viral replication by deletions in viral genes***

One approach to restrict viral replication specifically to tumor cells is by introducing genetic modifications that abrogate viral functions that are essential for replication in normal cells but are redundant in cancer cells. In most cases, this involves deletions in the *E1* region (Fig. 5B and C).

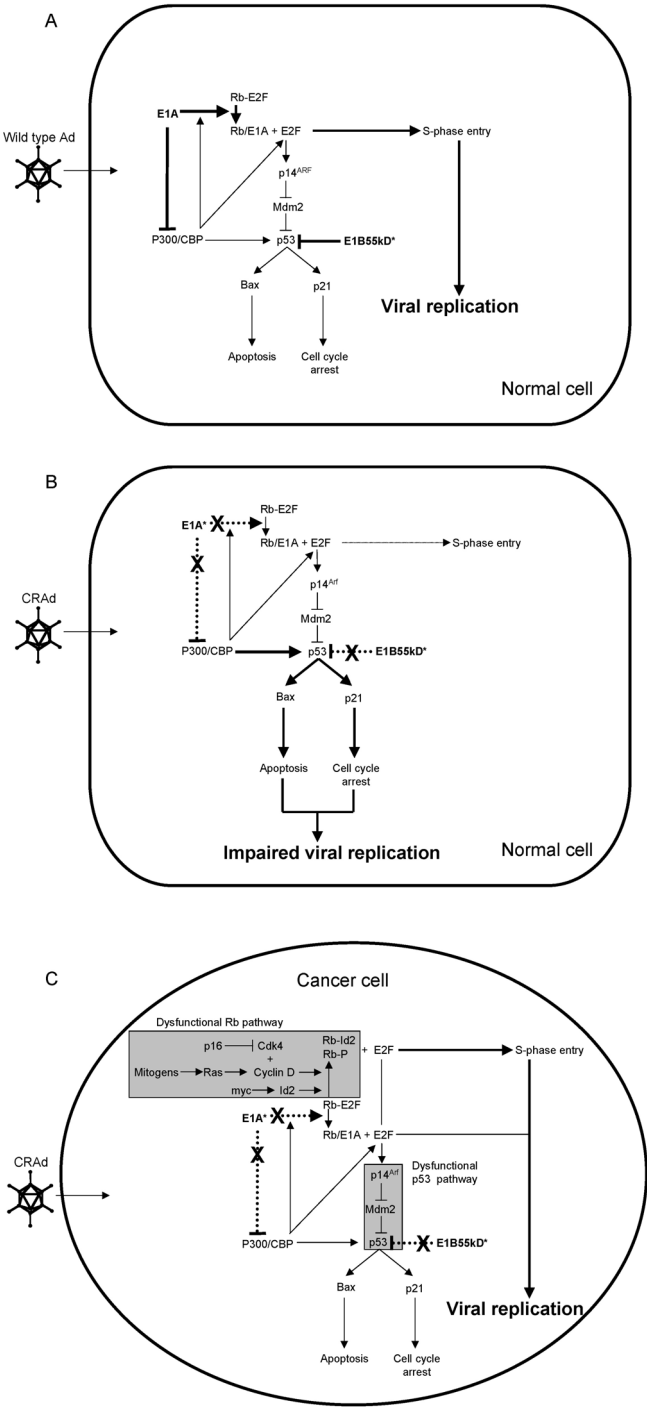



***Figure 5:*** *Schematic representation of the interaction between early adenovirus proteins and host cell proteins during replication of wild type adenovirus in normal cells (A) and CRAds carrying deletions in essential genes in normal cells (B) or in cancer cells (C). (A) E1A binds to pRb, causing dissociation of E2F. Free E2F can activate several cell cycle regulatory genes. This allows S-phase entry and virus replication in otherwise quiescent cells. Another function of E1A is to bind p300/CBP. This increases pRb binding affinity and decreases p53 transactivation functions. E1B55kD can bind directly to wild type p53 and prevents it from inducing apoptosis or cell cycle arrest. (B) Mutant E1A proteins incapable of binding pRb cannot induce S-phase and thus not promote adenoviral replication. Similarly, in normal cells with functional p53, p53-binding deficient E1B55kD fails to prevent induction of apoptosis or cell cycle arrest leading to impaired viral replication. (C) Replication of mutant viruses in malignant cells. The boxes represent cellular pathways that are frequently dysfunctional in cancer cells. For example, the Rb pathway is defective in cancer either through pRb deficiency, hyperphosphorylation or sequestration. As a result, E2F is continuously available for induction of S-phase progression and viral replication. Therefore, a deletion in the viral E1A gene that abrogates pRb binding does not hamper viral replication. The p53 pathway is also often dysfunctional in tumor cells ($p14^{art}$ deficiency, mdm2 amplification, p53 mutation). Inhibition of p53 by E1B55kD to prevent premature cell death by p53-induced of apoptosis is therefore not necessary in cancer cells.*

The by far most extensively studied CRAd is the mutant *dl*1520 (also known as ONYX-015) that contains a 827-base pair deletion in the *E1B-55kD* gene and a point mutation at nucleotide 2022, which results in abrogation of *E1B-55kD* expression (5). One of the functions of E1B-55kD is to bind the tumor suppressor protein p53 [151,152]. This binding inhibits p53 transcriptional activity and promotes the degradation of p53 [153]. *E1B-55kD* expression during the early phase of adenovirus replication temporarily inhibits p53-induced apoptosis to prevent premature cell death. *dl*1520 cannot inactivate p53 and the

virus was therefore expected to replicate only in cells lacking functional p53 [154]. In most, if not all, cancers p53 is dysfunctional. Over 50% of cancers contain an inactivating mutation in *p53* itself [130], whereas in the remaining cases p53 is efficiently degraded as a consequence of $p14^{ARF}$ deficiency, MDM2 amplification or viral protein expression; or p53 is sequestered in the cytoplasm [155-157]. Therefore, *dl*1520 should be widely applicable in cancer therapy. Originally, *dl*1520 was reported to selectively replicate in and kill cells with mutations in the *p53* gene [154]. Other reports, however, demonstrated a broader utility. For example, Heise *et al*. demonstrated that although normal human cells were highly resistant to *dl*1520, the virus replicated efficiently in numerous carcinoma cell lines with either mutant or normal *p53* gene sequences [158]. Two other reports also showed that the ability of *dl*1520 to replicate efficiently did not correlate with p53 status [159,160]. It was later shown that *dl*1520 could replicate in p53 wild type cancer cells with lost $p14^{ARF}$ expression and that reintroducing $p14^{ARF}$ into these cells suppressed replication [161,162]. Hence, the current view on *dl*1520 specificity is that this CRAd replicates in cells with dysfunctional p53, irrespective of the genetic defect causing this dysfunction.

Recently, Geoerger *et al.* reported in subcutaneous glioma xenograft models that intratumoral injections of *dl*1520 yielded significant tumor growth delay compared to control animals. Interestingly, wild type p53 status appeared to correlate with increased anti-tumor activity of *dl*1520 [163]. This finding was in agreement with the observation that the adenovirus life cycle is more rapid in p53 wild type cells, due to more effective cell lysis [164,165]. It disagreed, however, with the finding that the rapid adenovirus-induced lysis requires formation of a complex between p53 and E1B-55kD [166], because E1B-55kD is lacking in *dl*1520 infected cells. Hence, the interplay between adenovirus replication and p53 remains enigmatic.

An important disadvantage of *dl*1520 is that as a consequence of *E1B55kD* deletion, its oncolytic capacity is severely attenuated compared to wild type adenovirus. E1B55kD has important viral functions other than p53 inhibition, including promotion of viral mRNA transport and host cell protein synthesis shutoff. Therefore, more recently, a CRAd was constructed carrying a subtle single amino acid substitution in E1B-55kD that abolishes p53 binding, but leaves all other functions intact [167]. This CRAd replicated in cancer cells as efficiently as wild type adenovirus. Unfortunately, the paper did not report on selectivity of this CRAd for tumor cells.

Another type of CRAd based on a dysfunctional p53 pathway was developed by Ramachandra *et al*.[168]. This CRAd, 01/PEME, expresses a specific E2F antagonist, consisting of the pRb transrepression domain fused to the DNA binding domain of E2F, driven by a p53-responsive promoter. Furthermore, a deletion was introduced in *E1A* that abrogates sequestration of the p53 transcriptional co-activator p300/CBP. In cells with functional p53, 01/PEME expresses the E2F antagonist to inhibit S-phase entry and adenovirus *E1A* and *E2A* expression. 01/PEME did indeed not replicate in normal cells and replicated as efficiently as wild type adenovirus in cancer cells. In human xenograft tumor models, 01/PEME showed significantly enhanced efficacy compared to *dl*1520.

A different class of CRAds with abrogated viral function is based on mutations in E1A proteins that abolish binding to members of the Rb family of pocket proteins. pRb functions to modulate the cell cycle by regulating progression from G1 into S-phase. E1A binds to pRb and this results in release of the transcription factor E2F from pre-existing cellular E2F-pRb complexes. E2F can subsequently activate the adenovirus *E2* promoter as well as several cell cycle regulatory genes. This allows S-phase entry and virus replication in otherwise quiescent cells. Inactivation of the Rb family by E1A proteins and the binding domains involved were recently reviewed [169]. Paradigm examples of pRb-

binding deficient CRAds are AdΔ24 and *dl*922-947 that, although originally reported differently, both contain the same 24-bp deletion in the CR2 domain of the *E1A* gene, resulting in an E1A protein lacking amino acids 122 to 129 [170,171]. Although this has not been reported for these CRAds, the Δ24 mutation is expected to also abolish binding to the Rb family members p107 and p130. Because AdΔ24 and *dl*922-947 are defective in sequestering pRb from E2F, their replication depends on E2F being released through other means. This is the case in most, if not all cancer cells through pRb deficiency, pRb hyperphosphorylation, or pRb sequestration by cellular or viral proteins associated with malignancy. Therefore, while AdΔ24 and *dl*922-947 should be unable to replicate in quiescent normal tissues, their replication in cancer cells would not be hampered. Fueyo *et al.* demonstrated that AdΔ24 could replicate in and lyse both dividing and non dividing tumor cells with great efficiency whereas growth-arrested normal fibroblasts and cancer cells with restored pRb activity were resistant to the virus *in vitro*. Heise *et al*. showed that *dl*922-947 was effective against a range of cancer cells *in vitro* and *in vivo*. Its potency was superior to that of *dl*1520 and in some cancer types even to that of wild type adenovirus. However, they also found that, although *dl*922-947 was attenuated effectively in quiescent normal cells, it did replicate in proliferating normal cells [171]. This was, perhaps, not entirely unexpected as E2F is temporarily available free from pRb during normal regulated cell cycle progression.

In order to further increase the selectivity of *dl*922-947, Johnson *et al*. described the construction of the CRAd ONYX-411, that carries the *dl*922-947 mutation and expresses *E1A* and *E4* under control of the human *E2F-1* promoter [172]. This promoter is itself a transcriptional target of E2F. Thus, ONYX-411 is restricted to a single pathway defect (i.e., abundant free E2F) by a dual mechanism. This CRAd exhibited improved selectivity *in vitro* and reduced systemic toxicity in animal models *in vivo*.

Howe *et al*. increased the specificity of CRAds dependent on pRb pathway defects by combining two *E1A* deletions, i.e., Add*l*1101 and Add*l*1107 [173], creating E1Add*l*01/07 that lacks binding capacity for p300/CBP and pRb [174]. E1Add*l*01/07 should, therefore, be incapable of releasing E2F from pRb and of activating E2F by p300/CBP mediated acetylation. E1Add*l*01/07 was found defective for induction of cytopathic effects in dividing non-malignant cells compared to wild type adenovirus and the two single mutant viruses. Furthermore, in several different tumor models *in vivo*, the virus was effective in inhibiting tumor growth and extending the survival of the tumor bearing mice. Balagué *et al*. constructed the CRAd CB016 specifically for use against neoplasms associated with human papilloma virus (HPV), such as cervical cancers. CB016 combines the AdΔ24 mutation with a large deletion in the *E1A* gene encompassing the CR1 region, thereby inhibiting binding of E1A to p300/CBP, pRb and p107 [175]. The large deletion is assumed to also diminish transactivation of early adenovirus promoters by E1A proteins. In HPV associated cancers, HPV encoded E7 protein should complement for the lost E1A functions. Compared to wild type adenovirus and to AdΔ24, CB016 showed delayed replication in normal keratinocyte raft cultures. In contrast, CB016 replicated in and killed HPV *E6/E7* expressing keratinocytes effectively.

Very recently, a novel method was described for tumor selective replication of CRAds, based on an activated Ras pathway in cancer cells (Fig. 6) [176]. A common response of cells against a virus infection involves the interferon pathway. Interferon induces the expression of protein kinase R (*PKR*), a double stranded RNA-activated serine/threonine kinase. During adenoviral infection, viral dsRNAs resulting from bi-directional transcription of the two strands of the adenovirus genome can bind and activate PKR. Activated PKR phosphorylates eIF-2$\alpha$, leading to inhibition of protein synthesis

resulting in abrogation of viral replication. As a defense mechanism against this cellular antiviral response, adenoviruses produce virus associated (VA) RNAs that can also bind PKR, acting as PKR antagonists. Ad5 expresses two VA RNAs, of which VAI is the strongest PKR antagonist. In cancer cells, however, the interferon pathway is often defective. Furthermore, oncogenic Ras induces an inhibitor of PKR [177]. Based on this, it was hypothesized that in cancer cells with an activated Ras pathway, PKR inactivation by adenovirus VA RNA is redundant. Cascallo *et al*. demonstrated that VAI mutant adenovirus dl331 indeed exhibited reduced replication in cells with a normal Ras pathway, whereas introducing oncogenic Ras into these cells restored the replication of dl331 to wild type level. Accordingly, dl331 had anti-tumoral efficacy *in vivo* when administered intratumorally into xenografts of Ras-activated pancreatic cancer cells, with potency similar to wild type adenovirus. In contrast, in pancreatic tumor xenografts carrying wild type *Ras*, dl331 was less oncolytic than wild type adenovirus.

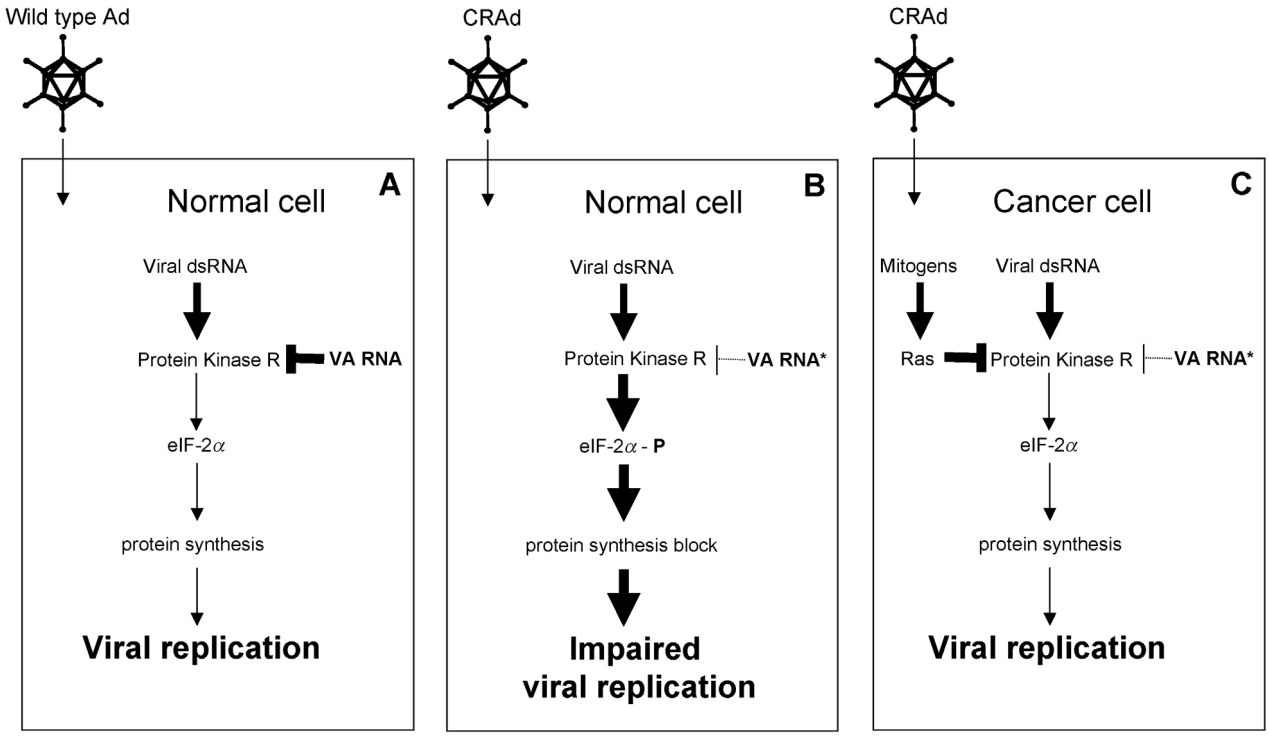



***Figure 6:*** *Schematic representation of the replication of wild type adenovirus in normal cells (A) and CRAds with deletions in Virus Associated (VA) RNAs in normal cells (B) or in cancer cells (C). In infected cells, adenoviral dsRNA transcripts are formed that can bind and activate protein kinase R (PKR). Via phosphorylation of eIF-2α (eIF-2α-P), activated PKR inhibits viral replication. As a defense mechanism, the virus produces VA RNAs that act as PKR antagonists (A). VA mutant virus cannot bind PKR and is therefore expected not to replicate in normal cells (B). Cancer cells, however, often have activated Ras that induces a PKR inhibitor, thereby allowing replication of the VA mutant adenovirus to ensue (C).*

Overall, genuine selective replication of CRAds has not been demonstrated yet. An explanation is that adenovirus exploits multiple pathways to promote viral and cellular DNA replication, with several virus proteins being capable of complementing each other. For example, E4orf6/7 was shown to displace pRb from E2F in the absence of E1A (34). Therefore, development of truly tumor selective CRAds will require introducing multiple mutations into the adenovirus genome. In this respect, Fukuda *et al*. recently presented a doubly restricted CRAd carrying mutant *E1A* and a deletion of *E1B-55kDa*, thus simultaneously targeting pRb and p53 pathway defects. This new CRAd replicated in and killed cancer cells as efficiently as a CRAd carrying only the *E1B-55kDa* deletion, but exhibited milder cytotoxicity [178].

***Limiting viral replication by tumor or tissue specific expression of essential adenovirus proteins***

Another way to limit viral replication to cancer cells is by transcriptionally regulating the expression of essential viral genes by using tumor or tissue specific promoters (fig. 7A and B). Several tissue specific CRAds have been developed in which the essential gene *E1A* is placed under the control of an exogenous promoter that is preferentially active in tumor cells. The first CRAd of this type was CN706 (later renamed CV706) that specifically replicates in prostate-specific antigen (PSA) expressing tumor cells [179]. In CN706, a minimal promoter derived from the human *PSA* gene drives expression of *E1A*. As intended, *E1A* was expressed at high levels in CN706-infected human PSA-producing LNCaP prostate cancer cells but not in CN706-infected PSA-negative prostate cancer cells. Consequently, CN706 replicated in LNCaP cells, but not in several other human cancer cell lines that did not express PSA. *In vivo*, LNCaP subcutaneous solid tumors growing in nude mice were destroyed by a single injection of CN706. To increase the specificity of CN706, two next generation CRAds were constructed that contained two different prostate-selective promoters to drive expression of *E1A* and *E1B*, i.e., the PSA promoter and the promoter/enhancer of the *hK2* gene or the rat probasin promoter [180,181]. These double-selective CRAds CV739 and CV764 exhibited an even higher therapeutic index of toxicity on PSA-positive prostate cancer cells over cells from other tissues. Neither CN706 nor CV764 could, however, eliminate distant pre-existent LNCaP xenograft tumors in mice following administration via the tail vein. In contrast, the CV739-derivative CV787 that retained the entire E3 region, which was lacking in the above-described CRAds exhibited 10-100 fold increased efficacy *in vitro* and *in vivo* [181]. Six weeks after an intravenous injection of CV787, prostate tumors growing in mice were reduced in size to less than 5% of their original size.

Matsubara *et al*. developed another CRAd, Ad-OC-E1a, that specifically replicated in both *PSA* expressing and non-expressing prostate cancer cells by using a non-collagenous bone matrix osteocalcin (OC) promoter to drive expression of *E1A*. OC is expressed in several solid tumors, including osteosarcoma and ovarian, lung, brain, and prostate cancers. All the prostate cancer cell lines tested, as well *PSA* expressing as non-expressing cell lines, were sensitive to Ad-OC-E1a induced cell lysis *in vitro*. Systemic administration of Ad-OC-E1a was effective against androgen-independent prostate cancer skeletal xenografts. Following repeated CRAd administration, tumors were eliminated completely [182].

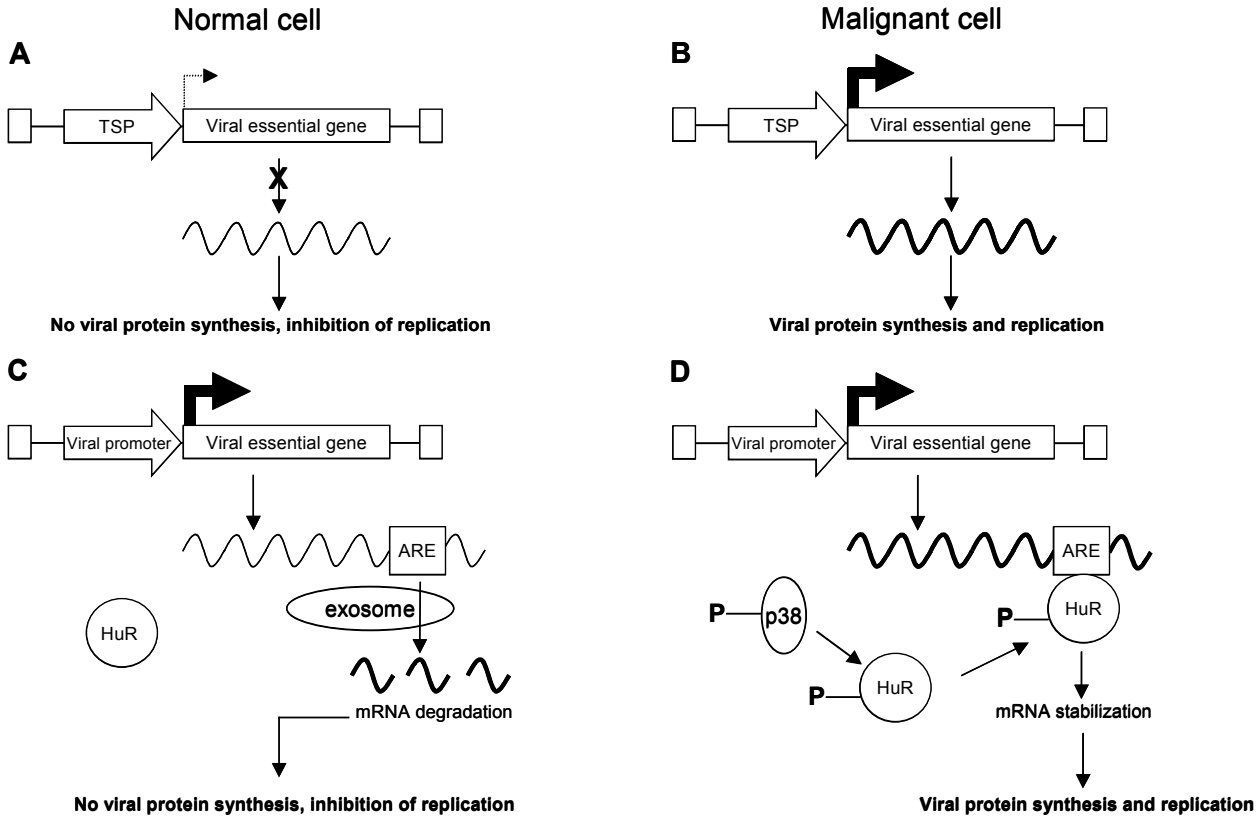



***Figure 7:*** *Schematic representation of the selective replication of CRAds in tumor cells by using a tumor specific promoter or by selective stabilization of mRNA . By regulating the expression of an essential viral gene, in particular E1A, via a tumor specific promoter (TSP) expression leading to replication will occur in malignant cells (B), but not in normal cells (A).*
*Selective stabilization of mRNAs of essential viral genes by introducing an AU-rich element (ARE) derived from the 3'UTR of one of many unstable mammalian mRNAs into their 3'UTR is illustrated in panels C and D. Several ARE-binding proteins (ABPs) were identified that have either a negative or a positive effect on mRNA stability. One of the ABPs that stabilizes their target mRNA is Hu-antigen R (HuR). HuR is depicted here by way of example, because it is known to be activated by p38/MAPK, which is frequently activated in tumor cells. In normal cells, HuR is not phosphorylated and activated, and can thus not bind to the ARE, leading to rapid mRNA degradation by the exosome, an enzymatic polyprotein complex that degrades mRNA. CRAds with the ARE sequence in the E1A 3'UTR will therefore not replicate in normal cells (C). In tumor cells with activated p38, HuR is activated (HuR-P) and can bind to the ARE thereby preventing mRNA degradation (D). The exact mechanism of mRNA stabilization is, however, not elucidated completely. An overview on current knowledge and hypotheses concerning this topic was recently given by Bevilacqua et al [183].*

Similar strategies have been explored to develop CRAds that specifically replicate in other types of cancer, like hepatocellular carcinoma using the $\alpha$-fetoprotein promoter [184,185], breast cancer using the *DF3/MUC1* gene promoter [186] or estrogen responsive element from the *pS2* gene promoter [187], melanoma using a tyrosinase enhancer/promoter construct [188-190], ovarian cancer using the *IAI.3B* promoter [191] or a truncated version of the L-plastin promoter [188] and lung cancer using the surfactant protein B promoter [192]. All these CRAds showed preferential replication in their target cells and demonstrated anti-tumor activity in preclinical models.

However, for many different human malignancies, tissue specific promoters have not been characterized yet. Moreover, the promoters used in these studies are primarily tissue-specific rather than tumor-specific. CRAds relying on these promoters might thus cause toxicity to normal tissues. To overcome this, general features that discriminate cancer cells from non-malignant cells have been explored to develop cancer specific CRAds.

Brunori *et al*. developed a colon cancer specific CRAd by taking advantage of the constitutive activation of the wnt pathway invariably seen in this type of cancer [193]. Activation of the wnt pathway leads to transactivation of promoters containing Tcf binding sites. Therefore, Tcf binding sites were placed in the adenoviral *E1B* and *E2* promoters resulting in decreased CRAd replication in non-permissive cell lines, whereas replication was comparable to wild type adenovirus in many colon cancer cell lines. Additionally inserting Tcf binding sites in the *E1A* promoter resulted in further improved specificity for colon cancer cells [194].

A common characteristic of all solid tumors is that they create an environment with low oxygen tension, hypoxia, due to their aberrant vasculature. The transcription factor hypoxia inducible factor (*HIF*) is expressed under hypoxic conditions, leading to expression of a number of genes needed for adaptation to the low oxygen situation via binding of HIF to hypoxia responsive elements (*HREs*) in the promoter of these genes. Hernandez-Alcoceba *et al*. described the construction of the CRAd AdEHT2, in which a minimal artificial promoter that contains HREs and estrogen responsive elements controls *E1A* expression [195]. AdEHT2 showed a good activation of *E1A* expression by hypoxia in different cancer cell lines or by estrogens in estrogen receptor expressing cell lines and this correlated with increased CRAd cytotoxicity. Cuevas *et al*. constructed a CRAd in which *E1A* expression is directed by an artificial minimal promoter containing nine tandem copies of the HRE and expression of *E4* is controlled by the *E2F-1* promoter [196]. This virus was as effective as wild type virus in eliminating cancer cells with increased HIF activity, and was severely attenuated in HIF-defective tumor cells and normal cells *in vitro*. Furthermore, intratumoral injection in renal cell carcinoma xenografts resulted in a significant reduction of tumor growth [196].

Another general characteristic of tumors is high expression of telomerase reverse tanscriptase (TERT). TERT is the catalytic subunit of the enzyme telomerase and is the rate-limiting determinant of enzymatic activity of human telomerase. Telomerase is a DNA polymerase, which directs the synthesis of TTAGGG (telomere) at the ends of chromosomes to compensate for telomere shortening during cell division. Telomerase is active in fetal development and becomes dormant in post-mitotic tissue soon after birth. High telomerase activity in tumor cells is one of the reasons for their immortality and telomerase is active in most human malignancies. CRAds based on this general feature of tumors could therefore be efficacious against a wide variety of tumors. The study by Hernandez-Alcoceba described above also included introduction of the *TERT* promoter into the *E4* region of AdEHT2. This CRAd, however, replicated in telomerase positive and negative cells, probably because *E1A* gene expression activated the *E4* gene directly (51). Other studies, however, described more successful use of the *TERT* promoter to regulate expression of *E1A* (53, 54). Huang *et al*. reported that a TERT-specific CRAd replicated in TERT positive cells as efficiently as wild type adenovirus, whereas replication was severely hampered in TERT-negative cells. *In vivo*, local administration into human hepatocellular carcinoma xenografts in nude mice resulted in significant inhibition of tumor growth as compared to control treated animals. In the study by Wirth *et al*. a smaller fragment of the TERT promoter was used to drive expression of *E1A*. Whereas the CRAd

replicated efficiently in several cancer cell lines, neither viral replication nor *E1A* expression was observed in human hepatocytes. To achieve higher levels of *E1A* expression, Kim *et al*. constructed a CRAd with a modified TERT promoter containing additional c-Myc and Sp1 binding sites. This virus induced cell killing as potently as the control virus *in vitro* and *in vivo*, but was approximately 100-1000 fold less cytotoxic to normal cells *in vitro* (Kim *et al*., 2003).

Many tumors have increased levels of the transcription factor *E2F*, either because of a deregulated Rb pathway or because of *E2F* gene amplification. Several CRAds have been developed that replicate specifically in cells with active E2F by placing the *E1A* gene under the *E2F* promoter. These CRAds replicated as efficiently as wild type virus in a panel of cancer cells, whereas normal cells were not capable of supporting CRAd replication [197,198].

A last general feature of solid tumors is that their growth requires new blood vessel formation. Targeting CRAds towards tumor vessels instead of tumor cells has the potential of depriving the tumor of its oxygen and nutrient supply and the advantage of a better delivery to the entire tumor. A number of genes, including *Flk-1* and *endoglin*, were shown to be overexpressed in angiogenic endothelial cells. Savontaus *et al*. constructed two CRAds, AdFlk-1 with *E1A* under the control of the *Flk-1* enhancer/promoter, and Ad.Flk-Endo that additionally has the *E1B* gene under the *endoglin* promoter. Both CRAds replicated efficiently in human umbilical vein endothelial cells, with replication of Ad.Flk-Endo being severely hampered in Flk-1 and endoglin negative cells [199].

For the design of CRAds with specific promoters it is important to consider that insertion into the adenovirus genome may affect selective expression of the gene, due to the presence of dominant transcriptional activators, as was observed for the *ERBB2* promoter [200]. When delivered in a plasmid, selective expression of the transgene in *ERBB2* expressing cells was observed, but this selectivity was lost when the expression cassette was delivered by replication deficient adenoviral vectors. Selective expression can, however, be retained by insulating the expression cassette from adenovirus expression elements by inserting transcription stop signals [201,202].

Recently, a novel method was described for development of CRAds based on selective mRNA stabilization in cancer cells (Fig. 7C and D) [203]. The expression of many proteins involved in early responses to certain physiological conditions, such as hypoxia, radiation exposure, inflammation and cell proliferation is regulated partly at the level of mRNA stability. The 3' UTRs of the mRNAs encoding these proteins contain destabilizing AU-rich elements (AREs), whose action is reversed under stress [204,205]. In tumor cells, continuous intracellular proliferative signals predominate, establishing conditions for selective stabilization of early-response-mRNAs. *PTGS2*, also known as *COX2*, is induced in different types of tumors and has been associated with a poor prognosis. *PTGS2* is induced partly by mitogen-activated protein kinase (MAPK)-dependent selective mRNA stabilization. MAPKs are downstream effectors of RAS-mediated transformation and receptor tyrosine kinase phosphorylation that are common in cancer. Ahmed *et al*. described the construction of the CRAd Ad-E1A-COX, in which a fragment encompassing AREs from *PTGS2* was introduced into the *E1A* 3'UTR. This resulted in destabilization of *E1A* mRNA and decreased *E1A* expression in normal cells. In contrast, Ad-E1A-COX was preferentially oncolytic in human tumor cells with high levels of active phosphorylated MAPK (P-MAPK) *in vitro*. Moreover, *in vivo* this CRAd was as effective as wild type virus in tumors with high P-MAPK activity, but generated no significant cytotoxic effects in tumors with low P-MAPK activity [203].

## LIMITED EFFECTS OF CRADS USED AS SINGLE AGENTS IN CLINICAL TRIALS

The safety and anti-tumor efficacy of *dl*1520 has been tested in several clinical trials in different types of tumors and using different administration routes. Following intratumoral injection, dose-limiting toxicities were not observed and maximally tolerated doses (MTDs) were not reached. The most common treatment associated toxicities were grade I-II flu-like symptoms, which did not correlate with viral dose [206-210]. Increases in neutralizing anti-adenovirus antibodies were commonly observed, but, importantly, these high neutralizing antibody titers did not appear to prevent CRAd replication in tumors [206]. In terms of therapeutic efficacy of CRAds as single agent, the most encouraging data were obtained by Nemunaitis *et al*. in 37 patients with recurrent head and neck cancer that were injected intratumorally or peritumorally with *dl*1520. A significant tumor regression was observed in 21% of evaluable patients, whereas no signs of virus were present in normal surrounding tissue, despite direct injection [206,211]. In one study, *dl*1520 was administered intraperitoneally [212]. Although also here the MTD was not reached, abdominal pain secondary to inflammation was common and in one patient dose-limiting. Intravascular administration of *dl*1520 to patients with colorectal carcinoma metastatic to the liver [213,214] or to patients with end-stage refractory carcinoma metastatic to the lung [215] was well tolerated at doses up to $2x10^{13}$ particles. Evidence of viral replication was observed in patients treated at high virus doses [214,215]. Also in these trials, the most common toxicities were mild flu-like symptoms. None of the studies with systemic *dl*1520 administration documented objective responses.

A phase I trial was also conducted with the CRAd CV706, that specifically replicates in PSA expressing prostate cancer cells [216]. Intra-prostatic CV706 injections appeared to be safe, because no CRAd-related grade III or IV toxicities were observed. The most common side effects were local pain and genitourinary symptoms. In this study, all patients demonstrated a dose-dependent reduction in PSA levels, suggesting CRAd efficacy.

Most importantly, clinical trials with CRAds have shown that administration of these viruses is a safe procedure without the manifestation of severe side effects. The lack of clinically significant toxicity in the liver is of particular importance, because most of the systemic administered adenoviruses end up in the liver. Unfortunately, no objective responses were documented with single agent therapy. Given this high degree of safety but inadequate efficacy, second generation viruses with greater potency will have to be engineered. In addition, combination therapies are being considered. These potential improvements will be discussed in the next sections.

## INCREASING THE EFFICACY OF VIROTHERAPY BY COMBINING CRADS WITH CONVENTIONAL THERAPIES

Given their limited efficacy in clinical trials, it is unlikely that CRAds will have a future as single agents in cancer treatment. In addition, conventional chemotherapy and radiotherapy have already significant, though incomplete, efficacy against many cancers. Therefore, patients entering clinical trials for new anti-cancer agents are usually not withheld conventional treatment. Furthermore, there is also a theoretical basis for additive or perhaps even supra-additive anti-cancer effects of CRAds combined with chemotherapy or radiotherapy. These considerations have directed investigations into the combination of

CRAds with conventional therapies. These studies have confirmed improved anti-tumor efficacies of combination treatments *in vitro*, *in vivo* and in clinical trials.

Chemotherapy has been combined with *dl*1520 and with liver- and prostate-specific CRAds. Heise *et al*. combined intratumoral *dl*1520 injection with cisplatin treatment and intravenous *dl*1520 infusion with 5-FU treatment in subcutaneous nude mouse carcinoma xenograft models [158]. Both combinations resulted in significantly increased responses compared to chemotherapy alone. This was extended by synergy observed between *dl*1520 and cisplatin plus 5-FU in three different cancer xenograft models [217]. Furthermore, in lung cancer cell lines and primary cultures *dl*1520 worked synergistically with paclitaxel and cisplatin [218]. Interestingly, synergy required that *dl*1520 was administered prior to or simultaneously with the drug. Although an explanation for this observation is currently lacking, it suggested that virus replication enhanced sensitivity to drugs rather than chemotherapy enhancing viral oncolysis. A mechanism potentially contributing to adenovirus-mediated chemosensitization is *E1A* expression, which has been shown to induce *p53* expression [149] as well as to render cells more sensitive to chemotherapy in a p53 independent manner [219]. Another possible result of adenovirus replication is the induction of chemosensitizing cytokines including TNF [220]. Li *et al*. described the combination of a hepatocellular carcinoma specific CRAd and doxorubicin treatment [221]. *In vitro* and *in vivo*, synergistic effects were observed which in the latter experiment resulted in complete elimination of subcutaneous Hep3B tumors four weeks after a single intravenous administration of both compounds. Similarly, Yu *et al*. tested the combination of CV787 with the chemotherapeutic drugs paclitaxel or docetaxel on prostate cancer cells [222]. *In vitro* and *in vivo*, a synergistic anti-cancer effect was observed on PSA-expressing prostate cancer cells when CV787 was combined with either of the two taxanes. PSA-positive prostate cancer cell specificity was retained in the combination treatment. In these experiments, the CRAd was administered simultaneously with or before or after taxane addition. Synergy was documented in all cases. Thus, the importance of correct scheduling reported for *dl*1520 was not confirmed for CV787 plus chemotherapy. Some insight into a possible mechanism for synergy of CRAd plus chemotherapy came from the observation that cells treated with the combination exhibited a greater burst size of CV787 compared to viral treatment alone. This suggested that the drugs speeded up the CRAd life cycle. A possible explanation for this effect is that many anticancer drugs, including etoposide, gemcitabine, topotecan and dexamethasone can increase adenovirus infection by increasing expression of the primary adenovirus receptor Coxsackie Adenovirus Receptor (CAR) on the cell surface [223]. This does, however, not provide a complete explanation, because paclitaxel was found not to induce CAR expression.

To date, *dl*1520 has been combined with chemotherapeutic drugs in four reported clinical trials. Khuri *et al*. described the results of a phase II trial in which *dl*1520 was injected intratumorally in combination with intravenous cisplatin and 5-FU in patients with recurrent squamous cell cancer of the head and neck [224]. In 19 of 30 (63%) evaluated patients an objective response was documented, with 8 patients showing a complete response and 11 a partial response, which was better than the responses seen in multi-center, randomized trials with 5-FU/cisplatin alone. No correlation was observed between response and baseline tumor size, baseline neutralizing antibody titer, *p53* gene status or prior treatment. The treatment was well tolerated; as for single-agent CRAd treatments, injection site pain and flu-like symptoms were seen. Nemunaitis *et al*. treated patients with lung metastases, with intravenous *dl*1520 injection and carboplatin and paclitaxel concurrently. Two patients showed tumor stabilization for more than 6 months [215]. Reid *et al*. combined *dl*1520 infusion into the hepatic artery with 5-FU and leucovorin. At high viral

doses, several anti-tumor responses were documented [214]. Finally, Hecht *et al*. injected pancreatic carcinomas with *dl*1520 under ultrasound guidance eight times over a two-month period, the last four injections combined with intravenous gemcitabine. While no objective responses were demonstrated after the first four CRAd injections, the combination treatment gave 10% objective partial regressions [225]. Although one should obviously be careful in comparing the outcome of different clinical studies, it seems fair to conclude that the efficacy obtained with CRAds plus chemotherapy is among the most promising results seen with CRAds so far. This warrants further development of this type of combination treatment.

Several reports have demonstrated the successful combination of cytolytic adenoviral therapy with radiotherapy (RT*) in vitro* and *in vivo*. Rogulski *et al*. tested the combination of *dl*1520 and RT on the colon carcinoma cell line RKO (p53 wild type) and the derivative cell line RKO.p53.13 that expresses an inactivating mutant of p53 [226]. *In vivo* experiments with subcutaneous xenografts demonstrated that in the parental cell line combination treatment with RT and *dl*1520 resulted in a tumor growth delay identical to that obtained with RT alone, consistent with the assumption that *dl*1520 is not effective in p53 wild type cells. In contrast, treatment of RKO.p53.13 tumors with *dl*1520 and RT produced an anti-tumor effect that was significantly greater than that achieved with either monotherapy. In contrast to these findings, a recent report by Georger *et al*. describes additive effects of *dl*1520 with RT in p53 mutant as well as in p53 functional subcutaneous human glioma xenografts *in vivo* [227]. Investigations into the potential mechanism of combined treatment effects did not provide a clear answer, but ruled out that irradiation increased adenovirus infection or replication. Induction of necrosis and apoptosis did not seem to play a major role either. Furthermore, Toth *et al*. found increased activities of CRAds with RT in cancer cell lines and could rule out radiation-induced replication as the possible mechanism [228]. These findings partially conflicted with another study using replication deficient adenoviruses, where it was found that irradiation enhanced virus uptake into cancer cells [229].

The PSA selective CRAd CV706 was shown to act synergistically with RT in LNCaP cells *in vitro* and *in vivo* [230]. In this study, in contrast to the observations described by Geoerger *et al*., increases in necrosis and apoptosis and a decreased blood vessel density were found in combination treated tumors. As was reported by the same group for the combination of CV787 with chemotherapy, CV706 plus RT combination increased viral burst size and did not affect the prostate specificity of the virus. Lamfers *et al*. demonstrated that the combination of RT and Ad5-Δ24RGD in primary glioma cell cultures resulted in cytotoxicity effects ranging from additive to supra-additive. To achieve the same therapeutic response *in vivo*, a 10-fold lower dose of Ad5-Δ24RGD was needed when it was combined with RT [231]. Taken together, it can be concluded that, although the mechanism remains unclear, perhaps because the two components are independent, the combination of CRAd with RT is promising for further preclinical and clinical evaluation.

## POSSIBLE WAYS TO MAKE CRADS MORE EFFECTIVE

### *Targeting CRAds towards tumor cells*

Almost all CRAds designed so far were derived from human adenovirus serotype 5 (Ad5). The infection of host cells by Ad5 is a two-step process. The first step is a high-affinity interaction of the knob domain of the adenovirus fiber protein with the cell surface receptor CAR [146,232]. Subsequent internalization, via receptor-mediated endocytosis, involves

interactions between the Arg-Gly-Asp (RGD) sequences of the adenovirus penton base proteins with cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. Unfortunately, many tumors exhibit limited levels of *CA*R expression [233-238], whereas *CAR* is abundantly expressed on many normal cell types. Douglas *et al*. demonstrated that CAR deficiency on tumor cells restricts the oncolytic potency of CRAds *in vitro* and *in vivo* [239]. Therefore, CAR deficiency emerges as a major limiting factor for effective use of CRAds for cancer gene therapy. A logical approach to circumvent inefficient CAR-mediated CRAd infection is by redirecting CRAd entry (i.e., targeting) via alternative cell surface molecules abundantly expressed on cancer cells. Modification of Ad tropism has so far primarily been studied in the context of replication deficient adenoviral vectors, but several strategies are now also being explored to target CRAds (Figure 8).

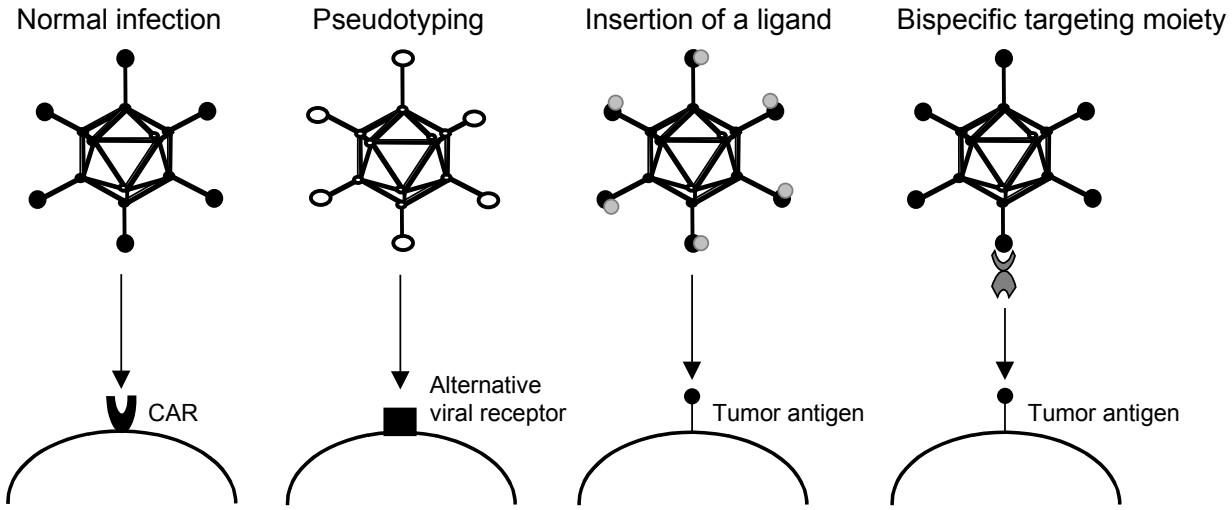



***Figure 8:*** *Schematic representation of different approaches to target CRAds to tumor cells. During native infection, adenovirus serotype 5 enters the cell following high-affinity binding to the cellular receptor CAR. Replacing the tropism-determining fiber knob domain of Ad5 with that of a different adenovirus serotype results in a virus with a modified tropism and improved transduction efficiency on several tumor types. Infectivity enhanced CRAds have also been constructed by inserting peptide motifs in the virus capsid that can bind to receptors on cancer cells. Furthermore, bispecific targeting moieties have been incorporated in CRAds. These molecules can bind on one side to the adenoviral fiber and on the other side to a tumor antigen.*

It has been known for long that various human adenovirus serotypes bind to distinct cell receptors (e.g.[240]). In addition, it appeared possible to replace the tropism-determining fiber knob domain of Ad5 with that of a different adenovirus serotype (e.g.[241,242]). Replication deficient Ad5/Ad3 chimeric viruses, i.e., vectors encapsidated in Ad5 virions carrying fibers consisting of the Ad5 tail and shaft domains and the Ad3 knob domain encompassing the receptor binding site, exhibited a modified tropism and improved transduction efficiency on several tumor types, including ovarian cancer, squamous cell carcinoma of the head and neck (SCCHN) and B-cell lymphomas [243-245]. Ad3 has an unidentified receptor distinct from that of Ad5 and therefore a different tissue tropism. Haviv *et al*. exploited Ad5/Ad3 chimeric fibers to target replication competent adenoviral vectors towards renal cell carcinoma cells deficient in *CAR* expression [246]. Ad5/3 virus successfully infected and replicated in renal cell carcinoma cancer cells that were resistant

to Ad5 infection. Kawakami *et al*. made the same comparison on SCCHN cells. They found a modest enhancement of infection of SCCHN cells by Ad5/3 compared to Ad5, but a much higher virus progeny production and oncolytic activity of the chimeric virus. Hence, the pseudotyping affected multiple steps of the adenovirus replication cycle, including post-infection processes [247]. Very recently, the first pseudotyped CRAd Ad5/3-Δ24 was reported. On primary ovarian cancer spheroids, this virus exhibited improved oncolysis compared to the control virus Ad5-Δ24. Moreover, in an intraperitoneal model of disseminated ovarian cancer, a single injection of Ad5/3-Δ24 increased the survival of tumor bearing mice significantly compared to injections with control viruses [248].

Obviously, the utility of pseudotyping is limited by the natural diversity of adenovirus receptor recognition. Defined targeting of cancer cell specific surface molecules requires synthetic design of targeted adenoviruses. For this, single- and two-component systems are being explored.

The design of single-component targeted adenovirus vectors by incorporating targeting ligands into adenovirus capsid proteins has been widely explored and was reviewed before [249]. The most extensively studied capsid protein modifications are peptide extension of the fiber protein carboxy-terminus and peptide insertion into the flexible fiber HI-loop. These modifications do not abrogate binding to CAR and do thus expand rather than target adenovirus entry. However, it should be possible to combine them with capsid protein mutations known to abolish native tropism [250] to construct truly targeted viruses.

In CRAds, two different targeting ligand incorporation strategies have been studied so far. Shinoura *et al*. described the construction and evaluation of the *E1B-55kD* deleted CRAd AdV-E1AdB-F/K20 carrying a stretch of 20 lysine residues at the COOH terminus of the fiber to allow CRAd binding to heparan sulfate cellular receptors. The infection and replication efficiency of AdV-E1AdB-F/K20 on glioma cells was greatly enhanced and its anti-tumor effect was much stronger compared to the parental control virus [251]. Suzuki *et al*. constructed Ad5-Δ24RGD, an infectivity-enhanced variant of AdΔ24 that contains a cyclic RGD peptide motif in the fiber HI-loop. Ad5-Δ24RGD exhibited enhanced propagation and oncolytic effect compared to the control virus AdΔ24 *in vitro* and *in vivo* [252]. Oddly, both Shinoura *et al*. and Suzuki *et al*. evaluated the oncolytic potency of their infectivity-enhanced CRAds only on CAR-positive tumor models. More recently, however, Ad5-Δ24RGD was tested on CAR-deficient rhabdomyosarcoma, ovarian carcinoma and glioma cells [231,238,253,254]. Ad5-Δ24RGD was found very effective in killing cancer cells *in vitro* and *in vivo*, the latter resulting in significant improvement in survival of tumor-bearing animals. Ad5-Δ24RGD was reported to be more oncolytic than the parental virus AdΔ24. However, the importance of targeting for effective CRAd treatment could not be established, because AdΔ24 lacks the E3 region, while Ad5-Δ24RGD carries an intact E3 region that includes the gene for Adenovirus Death Protein (ADP), which is involved in lysis of the infected cell [255]. Therefore, in a follow-up study, Suzuki *et al*. constructed AdΔ24 CRAds with and without E3 region and with and without RGD targeting sequence [256]. This allowed delineation of the effects exerted by the individual modifications. It was found that the superior potency of Ad5-Δ24RGD was primarily due to the incorporation of the E3 region, but contribution of the infectivity-enhancement to the anti-cancer effect was also confirmed.

Targeting by adenovirus capsid protein modification is bound by the structural demands on the incorporated ligand. So far, this approach has been limited to the use of small or flexible peptides. The versatility of tumor specific CRAd entry could potentially be much expanded if more complex ligands, such as antibodies, could be used. However,

such molecules have so far not been successfully incorporated as a structural component of the adenovirus capsid. In addition, the nuclear assembly of adenoviruses may preclude correct post-translational modification of protein moieties that are normally processed through the secretory pathway. For this reason, complex binding ligands including antibodies have so far only been successfully employed in two-component targeting strategies, where they were bound to the adenovirus fiber indirectly via a second protein moiety [134,233,257,258]. Also for CRAds it has been reported that infection of tumor cells in the presence of such a bispecific molecule is more efficient [259,260]. However, in such two-component strategies, the targeting moiety is not part of the CRAd genome and is thus lost upon viral replication. As a solution to this limitation, recently two AdΔ24 derived CRAds containing an expression cassette for a bispecific-targeting molecule were described. Hemminki *et al*. [261] utilized the targeting moiety sCAR-EGF, consisting of the extracellular domain of CAR fused to the Epidermal Growth Factor (EGF) that binds with specificity to the EGF receptor (EGFR) and we used 425-S11, a bispecific single chain Fv antibody fragment directed on one side to the adenovirus fiber and on the other side to EGFR [262]. As expected, AdΔ24-425S11 exhibited increased infection efficiency and replication on 2-D monolayers and 3-D tumor spheroids of CAR deficient cancer cells [262]. Surprisingly, incorporation of sCAR-EGF into the AdΔ24 backbone did not at all increase the oncolytic potency of the virus, but abrogated its cell killing potency [261]. The unexpected results with AdΔ24-sCAR-EGF appear to have been caused by the particular targeting ligand that was used. EGF is an active mitogen, with diverse regulatory functions related to cell growth, communication and development. Through an unknown mechanism, sCAR-EGF expression negatively influenced viral oncolysis as well as its own production [261]. Conversely, the anti-EGFR scFv that was used to construct AdΔ24-425S11 and that enhanced AdΔ24 oncolytic potency is an EGFR antagonist that binds to and neutralizes the receptor and has therefore no or perhaps even an inhibitory effect on cell processes related to EGFR signaling. These observations underscore the importance of thorough selection of the appropriate targeting moiety in designing targeted CRAds.

To generate a genuine tumor-targeted CRAd, it might perhaps be possible to combine the two-component targeting approach with mutations in the adenoviral genome to abrogate binding of the CRAd to CAR and integrins [263,264]. Such an approach is, however, not possible with targeting moieties based on sCAR, since such molecules bind specifically to the CAR-binding site in the fiber knob.

### *Combining CRAds with Gene-Directed Enzyme Prodrug Therapy*

GDEPT has been investigated extensively using replication deficient adenoviral vectors. The demonstrated synergy between CRAds and chemotherapy warranted studies into combinations of CRAds with the more selective GDEPT. Several investigators have attempted to enhance the anti-cancer efficacy of CRAds by incorporating *TK* into the CRAd genome and adding GCV. Wildner *et al*. constructed an *E1B-55kD* deleted CRAd expressing *TK* [265]. Intratumoral injection of this CRAd into human melanoma, cervical cancer or colon cancer xenografts in nude mice, followed by GCV treatment resulted in increased survival of the mice compared to viral treatment without prodrug administration or compared to replication deficient *TK* virus combined with GCV treatment [265,266]. Importantly, the combination of *TK* expressing CRAd plus GCV was only more effective than the CRAd alone when GCV was administered 3 days after CRAd injection. Simultaneous treatment was not beneficial, suggesting that GDEPT could interfere with CRAd replication. The same group also compared the efficacy of another *HSV-TK*

expressing *E1B-55kD* deleted CRAd with that of two other *E1B* positive *TK* expressing viruses in a subcutaneous lung cancer model and in an intraperitoneal ovarian cancer model [267]. In the subcutaneous model, GCV administration improved the oncolytic potency of the *E1B-55kD* deleted CRAd, but not of the other two much more potent viruses. Moreover, in the intraperitoneal model, the addition of GCV reduced instead of prolonged survival compared to virus treatment alone for all three viruses. Subsequent studies with *TK* expressing replication competent adenoviruses in a subcutaneous head and neck squamous cell carcinoma model [268,269] and subcutaneous and intraperitoneal lung cancer models [269] each showed that addition of GCV did not improve oncolytic potency. Thus, in all these models TK GDEPT appeared ineffective or even counterproductive in combination with replicating adenoviruses, despite the fact that in several experiments the first GCV injection was postponed until one week after virus injection. In contrast to these observations, Nanda *et al*. found that although TK/GCV treatment completely abrogated adenovirus replication when administered concomitantly, it significantly enhanced the oncolytic potency of an adenovirus *in vitro* and *in vivo* when GCV administration was started only one or two days after virus injection [270]. Apparently, there is a delicate balance between TK/GCV induced cell death and TK/GCV reduced cell kill by impaired viral replication. This makes TK/GCV GDEPT rather unattractive for use in combination with CRAds.

Another extensively studied GDEPT system utilizes the *E.coli* cytosine deaminase (CD) enzyme that converts 5-FC into the toxic drug 5-FU. Several studies have been performed in which a fusion gene consisting of *CD* and *TK* was inserted into a replication competent adenoviral vector, because double CD/TK suicide gene therapy is more efficacious than either GDEPT alone [271]. Freytag *et al*. constructed the *E1B-55kD* deleted CRAd FGR (subsequently renamed Ad5-CD/TK*rep*) expressing the *CD/TK* fusion gene [272]. FGR was quite effective in killing cancer cells *in vitro* when combined with either prodrug, which effect could be further augmented by irradiation. Interestingly, these effects occurred at prodrug concentrations that effectively inhibited adenovirus replication, i.e., under combinations where the CRAd functioned as a GDEPT expression vector rather than an oncolytic agent. This suggests that the assumed CRAd plus GDEPT combination effect was in fact an adenovirus protein plus GDEPT effect. This fits quite well with the known synergy between adenovirus *E1A* expression and chemotherapy [219] and explains why the GDEPT/CRAd combination seems to work best for rather weak CRAds that may act more or less as replication deficient *E1A* expression vectors. *In vivo* studies with FGR in subcutaneous cervical carcinoma tumors and treatment with the two prodrugs resulted in a remarkable reduction in tumor volume compared to mice treated with virus only, or in combination with one prodrug, suggesting synergistic interactions between the suicide systems. Combining the double suicide systems with RT could even further enhance the efficacy of this approach [271]. However, in two *in vivo* prostate cancer models, the addition of double prodrug therapy did not improve tumor control beyond that of FGR viral therapy [273]. Nevertheless, these results led to a phase I study for the treatment of locally recurrent prostate cancer in 16 patients [57]. The virus was delivered intraprostatically followed 2 days later with 5-FC and GCV treatment. Escalation up to $10^{12}$ viral particles and two weeks of GV and 5-FC treatment did not result in dose limiting toxicities and the MTD was not defined, indicating that this approach can be safely applied to humans. Forty-four percent of the evaluated patients showed a decrease in PSA level indicative of tumor regression of more than 25%, with 19% exhibiting a more than 50% reduction. Two patients were negative for adenocarcinoma by biopsy 1 year after the treatment.

A last GDEPT system that was very recently explored in CRAds uses the CE enzyme that activates the prodrug CPT-11 into the toxic drug SN-38. Stubdal *et al*. incorporated the rabbit *CE* gene into the *dl*1520 genome. *In vitro*, in the presence of CPT-11 *CE*-expressing *dl*1520 derived viruses exhibited increased toxicity on a colon carcinoma cell compared to *dl*1520. In subcutaneous colon cancer xenografts growing in nude mice treatment with *CE*-expressing CRAds and CPT-11 enhanced the survival of these mice [274].

All together, results from preclinical and clinical studies indicate that CRAd plus GDEPT combination treatment is showing signs of improved efficacy warranting further investigation. However, careful evaluation of CRAd efficacy enhancing versus abrogating activities of enzyme/prodrug systems is required to select synergistic CRAd/GDEPT combinations. Since the aim of such endeavors is to enhance the efficacy of CRAd potency, in our view these investigations should focus on testing GDEPT systems in the context of the stronger CRAd types.

***Insertion of therapeutic genes into the CRAd genome***

Finally, CRAds could be developed into more powerful anticancer agents by inserting therapeutic genes into their genome (summarized in figure 9). There are two good reasons to express a transgene in a CRAd. First, the gene product can be used to enhance the inherent oncolytic property of the CRAd to realize its full anti-cancer potential. Second, the CRAd can be used as a very potent expression vector for an anti-cancer therapeutic. This concept has enormous potential, as the expression cassette for the therapeutic agent is amplified over 1000-fold in each infected cell.

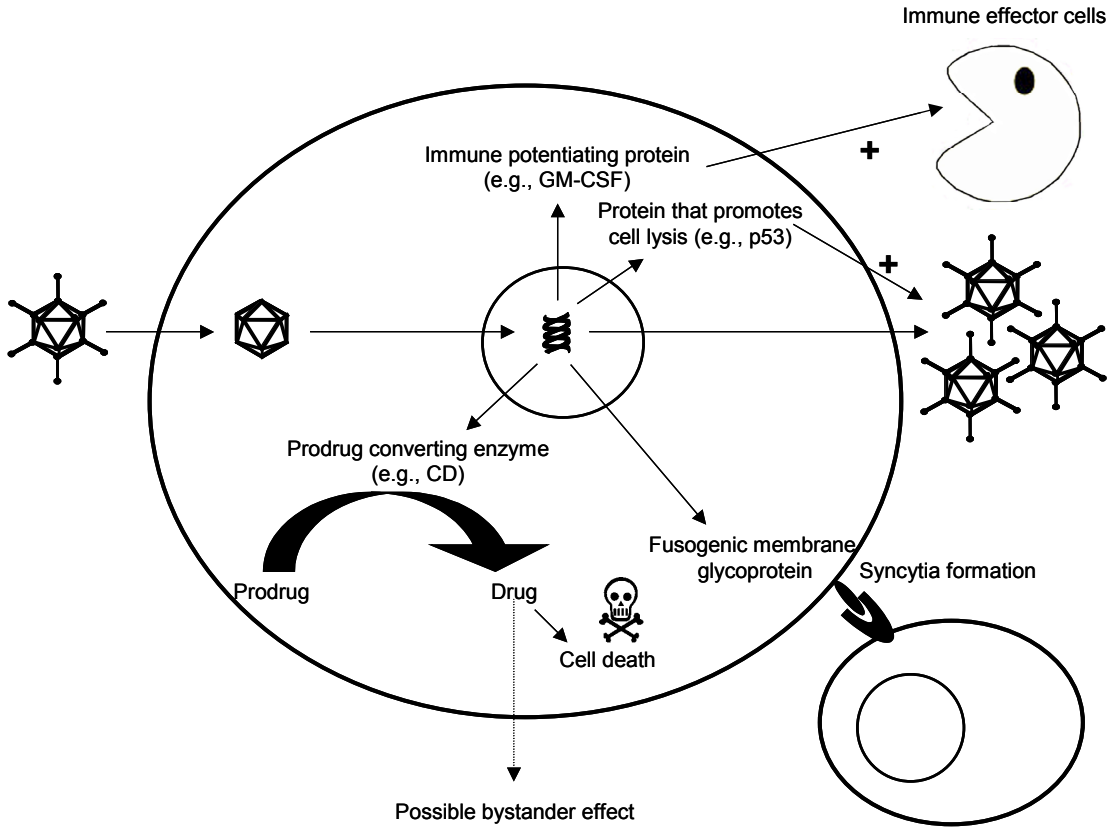



***Figure 9:*** *Schematic representation of different strategies to increase the anti-tumor efficacy of CRAds by incorporation of therapeutic genes. See text for details.*

The first approach aims to enhance the inherent cancer cell killing potency of the CRAd. Besides entry of CRAds into tumor cells, lysis of infected cells is another critical step that determines the efficacy of CRAd-based therapy. The E3-encoded ADP promotes lysis of infected cells and is expressed at very late stages of viral infection [144,255]. By comparing the CRAd CV787 with intact *E3* region to the otherwise identical CRAd CV739 lacking *E3*, the important contribution of E3 proteins to the anti-tumor efficacy *in vitro* and *in vivo* was demonstrated [181]. CRAds overexpressing *ADP* were reported to lyse and spread from cell to cell more rapidly than wild type adenovirus, resulting in improved *in vivo* anti-cancer effect [275]. Suzuki *et al*. compared *E3*-positive and *E3*-negative variants of AdΔ24 and the integrin targeted derivative Ad5-Δ24RGD [256]. The presence of the *E3* region enhanced the spreading ability of the CRAd resulting in superior oncolytic potency compared with *E3* negative CRAds *in vitro* and *in vivo*. The mechanism through which ADP enhances the viral spread is not elucidated yet. However, the effect of ADP is clearly not tumor selective. Therefore, investigators have sought to achieve increased tumor selective cell lysis by insertion of therapeutic genes other than *ADP*. These studies have focused on cell death pathways that are defective in cancer cells. Mi *et al*. demonstrated that insertion of a dominant-negative *I-κB* that sensitizes cells to Tumor Necrosis Factor (TNF)-induced apoptosis in an adenovirus enhanced viral release when apoptosis was induced after virion assembly was completed [276]. In contrast, premature cell death during viral DNA replication compromised virus production, emphasizing the importance of careful timing of apoptosis induction. One way to accomplish this is to express a pro-apoptotic gene that is regulated during replication by the CRAd itself. In this regard, two studies have explored the effects of inserting p53 into the genome of a CRAd [277] or replication competent adenovirus [278] to improve viral burst specifically in tumor cells. We described the construction of AdΔ24-p53 in which a constitutive *p53* expression cassette was inserted in place of the *E3* region [277]. AdΔ24-p53 exhibited enhanced oncolytic potency compared to the control vector AdΔ24 on a vast majority of tested cancer cell lines. As expected, the observed effects were most pronounced on p53 deficient cells. However, there was no significant correlation found with the p53 functional status of the cell. Exogenous *p53* expression also augmented CRAd potency in several cell lines where this was less expected, because these cells express p53-inhibiting proteins or p53 dominant-negative mutants. Recently, we confirmed that AdΔ24-p53 is also more potent than its parent in killing primary cancer cells from many different patient specimens and in inhibiting tumor growth in three different cancer xenograft models *in vivo* [279]Its). Sauthoff *et al*. expressed *p53* during the late replication phase of an adenovirus without E3 region [278]. They found that the *p53*-expressing virus in comparison to a wild type adenovirus expressing ADP was more cytotoxic to cancer cells but less cytotoxic to normal cells, suggesting that this modification may contribute to improving CRAd selectivity.

The second approach combines CRAd treatment with anti-cancer gene therapy. The CRAd/GDEPT treatment discussed above is an example of this approach. In addition, several other cancer therapeutic genes have been evaluated in the context of CRAds. For example, CRAds have been used to deliver therapeutic genes that activate the immune system. Interferon plays an important role in a multitude of immunological processes, among them the upregulation of MHC class I and class II. Human breast cancer cells transduced with the interferon gene failed to grow when transplanted into nude mice. Therefore, Zhang *et al*. constructed a replication competent adenoviral vector carrying the human interferon gene in the *E3* region [280]. Two cell lines grown as tumors in nude mice completely or partially regressed after injections with this virus, with wild type adenovirus being less effective. Kurihara *et al*. constructed a breast cancer specific CRAd expressing

*TNF*, known to have selective anti-tumor activity [186]. Nude mice bearing subcutaneous breast cancer xenografts were treated with this virus or with the control virus expressing *GFP* instead of *TNF*. Treatment with the TNF-CRAd but not with the control virus was associated with regression to barely palpable tumors that sustained long-term. Bristol *et al*. constructed a CRAd in which *GM-CSF*, a potent inducer of specific, long-lasting anti-tumoral immunity, was incorporated into the viral genome and its expression was controlled by the adenoviral E3 promoter [281]. In two xenograft models, in which the CRAd was injected intratumorally, GM-CSF was detected in serum and tumor extracts, resulting in a significantly enlarged anti-tumor response compared to untreated mice or mice treated with replication deficient adenovirus. Compared to a replication competent control virus that lacks the *GM-CSF* expression cassette, in one of the tumor models a significant difference in tumor growth was observed. Very recently, two genes in the *E3* region of a CRAd were replaced with *TNF* and *monocyte chemotactic protein-3*, respectively [282]. The effect of this virus on the immunological response remains to be determined.

Another strategy to enhance the efficacy of CRAds is by inducing cell killing through induction of fusion of tumor cells to form large multinucleated syncytia by fusogenic membrane glycoproteins (FMG). The formation and subsequent disintegration of syncytia is also immunostimulatory [283]. Li *et al*. showed that an adenovirus expressing the fusogenic envelope protein of human immunodeficiency virus (HIV) induced syncytium formation in infected cell cultures expressing HIV receptors. Importantly, syncytium formation elevated adenovirus production and release [284]. Recently, Ahmed *et al*. demonstrated that virotherapy in combination with injection of a plasmid encoding the Gibbon Ape Leukemia Virus (GALV) hyperfusogenic envelope FMG effectively treated large established tumors at doses of plasmids or virus that alone were ineffective [285]. Inserting the *GALV-FMG* into a CRAd genome seems therefore an interesting option. Many other useful transgenes, that inhibit cell proliferation, migration, invasion, or blood vessel formation, can be envisaged, but have so far not been evaluated in replicating adenoviruses. For some therapeutic transgenes, it may be required that expression is temporally regulated, to avoid interference with CRAd replication. Several ways to express transgenes only during the late phase of replication were reported. For example, Sauthoff *et al*. used an Internal Ribosome Entry Site to link transgene expression to the fiber expression unit [278] and Hawkins and Hermiston replaced the *ADP* open reading frame with the transgene coding sequences [286]. Thus, methods are in place to also use transgenes that may not only be detrimental to cancer cells but also to the virus.

All together, enhancing the oncolytic potency of CRAds by inserting genes encoding therapeutic proteins that are specifically active in tumor cells is very useful for further development of CRAds into clinically applicable tools for the treatment of cancer.

## OUTLINE OF THE THESIS

The major goal of this thesis was to improve the efficacy of chemotherapy with CPT-11, an approved anti-cancer agent for treatment of colorectal cancer, by selective activation of the prodrug at the site of the tumor. In the general introduction in ***Chapter 1*** background information is given on enzyme prodrug therapy with replication deficient adenoviral vectors, with emphasis on the carboxylesterase (CE)/CPT-11 system.

GDEPT with CPT-11 and CE to convert the prodrug CPT-11 into the toxic drug SN-38 has several advantages not offered by other enzyme prodrug models. First, CPT-11 has demonstrated considerable anti-tumor activity as a single agent, indicating an intrinsic sensitivity of several types of solid tumors to this drug. Second, xenograft experiments showed that CPT-11 has a steep dose-response curve, suggesting that even a relatively modest increase in drug activation in tumor cells could produce remarkable increases in anti-tumor activity. Third, the molecular target of SN-38 is topoisomerase I, an essential enzyme, in which no resistance-conferring mutations have been identified in tumors of patients undergoing treatment with camptothecins. Fourth, since the basis for substrate specificity of most human CEs is known, more suited prodrugs based on existing or new classes of anti-tumor agents, or enzymes with a higher specific activity for CPT-11, could be designed. Fifth, SN-38 freely passes through cell membranes, thereby increasing the likelihood that a bystander effect might occur.

Although CPT-11 therapy is effective against colon cancer, only 5% of intravenously administered CPT-11 is converted into SN-38. With GDEPT, it is possible to increase the levels of CE at the site of the tumor, resulting in increased conversion of the prodrug into the drug. This should lead to increased efficacy of the chemotherapy whereas toxicity is reduced.

In this thesis, we focus on developing adenoviral vectors with expression cassettes encoding different forms of the prodrug converting enzyme CE for the treatment of colorectal cancer. We envisioned that expression of a secreted form of human liver *CE2* could increase the bystander effect, since extracellularly formed SN-38 might more easily diffuse through a solid tumor mass, resulting in toxicity to untransduced neighboring cells. A secreted, tumor-targeted form could, furthermore, prevent leakage of the enzyme into the circulation, thereby decreasing the possibility that of side effects occur. To investigate this, we describe in ***Chapter 2*** the construction and characterization of secreted and secreted, targeted forms of human liver CE2. Secreted CE2 was obtained by deletion of a cellular C-terminal retention signal and introduction of a secretion signal. The targeted fusion protein consisted of the secreted form and a scFv antibody directed to the tumor antigen Epithelial Cell Adhesion Molecule (EpCAM). Delivery of these enzymes to the tumor can be achieved by transduction of tumor cells with these cDNA constructs. To that end, the genes encoding secreted or secreted, EpCAM targeted CE2 were cloned into replication deficient adenoviral vectors. The construction of the replication deficient adenoviral vector containing the gene encoding the targeted fusion protein is described in ***Chapter 3***. In 3-dimensional colon cancer spheroids, we determine the penetration capacity of the fusion protein and the therapeutic efficacy of this replication deficient adenovirus in combination with CPT-11 treatment. In ***Chapter 4***, the construction of a replication deficient adenoviral vector expressing the secreted form of *CE2* is described and the utility of this virus in combination with CPT-11 for the treatment of osteosarcoma cell lines or primary cell cultures *in vitro* and *in vivo* is evaluated.

Another way to overcome the poor penetration capacity of the adenoviral vectors is by using conditionally replicating adenoviruses (CRAds) that selectively replicate in tumor

cells. The general introduction in ***Chapter 1*** also gives an overview on the working mechanisms of conditionally replicating adenoviruses (CRAds), the efficacy of CRAds in clinical trials and possible ways to improve the efficacy of CRAds. An example of such a CRAd is Ad5-Δ24.E3. In ***Chapter 5***, we describe the construction of an Ad5-Δ24.E3 derivative expressing the secreted form of *CE2* and its applicability for the treatment of colon cancer in combination with chemotherapy with CPT-11. In ***Chapter 6***, we compare in colon cancer cell lines the oncolytic potency of the CRAd Ad5-Δ24 with its derivative Ad5Δ24-p53 that expresses functional *p53* upon infection. Furthermore, we investigate whether the efficacy of Ad5Δ24-p53 could be further improved by treatment with sub-toxic dose of the chemotherapeutic drug oxaliplatin.

Finally, in ***Chapter 7***, the results are summarized, discussed and put in current perspective.

## REFERENCES


1 Scheithauer W *et al.* Randomised comparison of combination chemotherapy plus supportive care with supportive care alone in patients with metastatic colorectal cancer. *Bmj 1993*; **306:** 752-755.

2 Friedmann T, Roblin R. Gene therapy for human genetic disease? *Science 1972*; **175:** 949-955.

3 Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res 1986*; **46:** 5276-5281.

4 Freeman SM, Ramesh R, Marrogi AJ. Immune system in suicide-gene therapy. *Lancet 1997*; **349:** 2-3.

5 Caruso M *et al.* Regression of established macroscopic liver metastases after in situ transduction of a suicide gene. *Proc Natl Acad Sci U S A 1993*; **90:** 7024-7028.

6 Vile RG, Diaz RM, Castleden S, Chong H. Targeted gene therapy for cancer: herpes simplex virus thymidine kinase gene-mediated cell killing leads to anti-tumour immunity that can be augmented by co-expression of cytokines in the tumour cells. *Biochem Soc Trans 1997*; **25:** 717-722.

7 Consalvo M *et al.* 5-Fluorocytosine-induced eradication of murine adenocarcinomas engineered to express the cytosine deaminase suicide gene requires host immune competence and leaves an efficient memory. *J Immunol 1995*; **154:** 5302-5312.

8 Yamamoto S *et al.* Herpes simplex virus thymidine kinase/ganciclovir-mediated killing of tumor cell induces tumor-specific cytotoxic T cells in mice. *Cancer Gene Ther 1997*; **4:** 91-96.

9 Kuriyama S *et al.* Cancer gene therapy with HSV-tk/GCV system depends on T-cell-mediated immune responses and causes apoptotic death of tumor cells in vivo. *Int J Cancer 1999*; **83:** 374-380.

10 Culver KW *et al.* In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science 1992*; **256:** 1550-1552.

11 O'Malley BW, Jr., Chen SH, Schwartz MR, Woo SL. Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res 1995*; **55:** 1080-1085.

12 Smythe WR *et al.* Treatment of experimental human mesothelioma using adenovirus transfer of the herpes simplex thymidine kinase gene. *Ann Surg 1995*; **222:** 78-86.

13 Freeman SM *et al.* The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res 1993*; **53:** 5274-5283.

14 Elshami AA *et al.* Gap junctions play a role in the 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system in vitro. *Gene Ther 1996*; **3:** 85-92.

15 Dilber MS *et al.* Gap junctions promote the bystander effect of herpes simplex virus thymidine kinase in vivo. *Cancer Res 1997*; **57:** 1523-1528.

16 Touraine RL, Vahanian N, Ramsey WJ, Blaese RM. Enhancement of the herpes simplex virus thymidine kinase/ganciclovir bystander effect and its antitumor efficacy in vivo by pharmacologic manipulation of gap junctions. *Hum Gene Ther 1998*; **9:** 2385-2391.

17 Mesnil M, Yamasaki H. Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. *Cancer Res 2000*; **60:** 3989-3999.

18 Colombo BM *et al.* The "bystander effect": association of U-87 cell death with ganciclovir-mediated apoptosis of nearby cells and lack of effect in athymic mice. *Hum Gene Ther 1995*; **6:** 763-772.
19 Gagandeep S *et al.* Prodrug-activated gene therapy: involvement of an immunological component in the "bystander effect". *Cancer Gene Ther 1996*; **3:** 83-88.
20 Wilson KM *et al.* HSV-tk gene therapy in head and neck squamous cell carcinoma. Enhancement by the local and distant bystander effect. *Arch Otolaryngol Head Neck Surg 1996*; **122:** 746-749.
21 Oldfield EH *et al.* Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. *Hum Gene Ther 1993*; **4:** 39-69.
22 Eck SL *et al.* Treatment of advanced CNS malignancies with the recombinant adenovirus H5.010RSVTK: a phase I trial. *Hum Gene Ther 1996*; **7:** 1465-1482.
23 Izquierdo M *et al.* Human malignant brain tumor response to herpes simplex thymidine kinase (HSVtk)/ganciclovir gene therapy. *Gene Ther 1996*; **3:** 491-495.
24 Ram Z *et al.* Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med 1997*; **3:** 1354-1361.
25 Stockhammer G *et al.* Gene therapy for glioblastoma [correction of gliobestome] multiform: in vivo tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. *J Mol Med 1997*; **75:** 300-304.
26 Klatzmann D *et al.* A phase I/II study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for recurrent glioblastoma. Study Group on Gene Therapy for Glioblastoma. *Hum Gene Ther 1998*; **9:** 2595-2604.
27 Shand N *et al.* A phase 1-2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. GLI328 European-Canadian Study Group. *Hum Gene Ther 1999*; **10:** 2325-2335.
28 Klatzmann D *et al.* A phase I/II dose-escalation study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for metastatic melanoma. Study Group on Gene Therapy of Metastatic Melanoma. *Hum Gene Ther 1998*; **9:** 2585-2594.
29 Herman JR *et al.* In situ gene therapy for adenocarcinoma of the prostate: a phase I clinical trial. *Hum Gene Ther 1999*; **10:** 1239-1249.
30 Shalev M *et al.* Suicide gene therapy for prostate cancer using a replication-deficient adenovirus containing the herpesvirus thymidine kinase gene. *World J Urol 2000*; **18:** 125-129.
31 Balzarini J, Bohman C, Walker RT, de Clercq E. Comparative cytostatic activity of different antiherpetic drugs against herpes simplex virus thymidine kinase gene-transfected tumor cells. *Mol Pharmacol 1994*; **45:** 1253-1258.
32 Balzarini J *et al.* Superior cytostatic activity of the ganciclovir elaidic acid ester due to the prolonged intracellular retention of ganciclovir anabolites in herpes simplex virus type 1 thymidine kinase gene-transfected tumor cells. *Gene Ther 1998*; **5:** 419-426.
33 Degreve B, De Clercq E, Balzarini J. Bystander effect of purine nucleoside analogues in HSV-1 tk suicide gene therapy is superior to that of pyrimidine nucleoside analogues. *Gene Ther 1999*; **6:** 162-170.
34 Thust R *et al.* Cytogenetic genotoxicity of anti-herpes purine nucleoside analogues in CHO cells expressing the thymidine kinase gene of herpes simplex virus type 1:

comparison of ganciclovir, penciclovir and aciclovir. *Mutagenesis 2000*; **15:** 177-184.

35 Kokoris MS, Sabo P, Adman ET, Black ME. Enhancement of tumor ablation by a selected HSV-1 thymidine kinase mutant. *Gene Ther 1999*; **6:** 1415-1426.

36 Kokoris MS, Sabo P, Black ME. In vitro evaluation of mutant HSV-1 thymidine kinases for suicide gene therapy. *Anticancer Res 2000*; **20:** 959-963.

37 Austin EA, Huber BE. A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of Escherichia coli cytosine deaminase. *Mol Pharmacol 1993*; **43:** 380-387.

38 Ge K *et al.* Transduction of cytosine deaminase gene makes rat glioma cells highly sensitive to 5-fluorocytosine. *Int J Cancer 1997*; **71:** 675-679.

39 Mullen CA, Coale MM, Lowe R, Blaese RM. Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor. *Cancer Res 1994*; **54:** 1503-1506.

40 Huber BE *et al.* In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res 1993*; **53:** 4619-4626.

41 Huber BE *et al.* Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci U S A 1994*; **91:** 8302-8306.

42 Ohwada A, Hirschowitz EA, Crystal RG. Regional delivery of an adenovirus vector containing the Escherichia coli cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum Gene Ther 1996*; **7:** 1567-1576.

43 Kanai F *et al.* In vivo gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. *Cancer Res 1997*; **57:** 461-465.

44 Ichikawa T *et al.* In vivo efficacy and toxicity of 5-fluorocytosine/cytosine deaminase gene therapy for malignant gliomas mediated by adenovirus. *Cancer Gene Ther 2000*; **7:** 74-82.

45 Topf N, Worgall S, Hackett NR, Crystal RG. Regional 'pro-drug' gene therapy: intravenous administration of an adenoviral vector expressing the E. coli cytosine deaminase gene and systemic administration of 5-fluorocytosine suppresses growth of hepatic metastasis of colon carcinoma. *Gene Ther 1998*; **5:** 507-513.

46 Pandha HS *et al.* Genetic prodrug activation therapy for breast cancer: A phase I clinical trial of erbB-2-directed suicide gene expression. *J Clin Oncol 1999*; **17:** 2180-2189.

47 Germano IM, Fable J, Gultekin SH, Silvers A. Adenovirus/herpes simplex-thymidine kinase/ganciclovir complex: preliminary results of a phase I trial in patients with recurrent malignant gliomas. *J Neurooncol 2003*; **65:** 279-289.

48 Prados MD *et al.* Treatment of progressive or recurrent glioblastoma multiforme in adults with herpes simplex virus thymidine kinase gene vector-producer cells followed by intravenous ganciclovir administration: a phase I/II multi-institutional trial. *J Neurooncol 2003*; **65:** 269-278.

49 Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther 2000*; **11:** 2389-2401.

50 Shalev M *et al.* Suicide gene therapy toxicity after multiple and repeat injections in patients with localized prostate cancer. *J Urol 2000*; **163:** 1747-1750.

51 Teh BS *et al.* Phase I/II trial evaluating combined radiotherapy and in situ gene therapy with or without hormonal therapy in the treatment of prostate cancer--a preliminary report. *Int J Radiat Oncol Biol Phys 2001*; **51:** 605-613.

52 Teh BS *et al.* Phase I-II trial evaluating combined intensity-modulated radiotherapy and in situ gene therapy with or without hormonal therapy in treatment of prostate cancer-interim report on PSA response and biopsy data. *Int J Radiat Oncol Biol Phys 2004*; **58:** 1520-1529.

53 Sung MW *et al.* Intratumoral adenovirus-mediated suicide gene transfer for hepatic metastases from colorectal adenocarcinoma: results of a phase I clinical trial. *Mol Ther 2001*; **4:** 182-191.

54 Treat J *et al.* Treatment of advanced mesothelioma with the recombinant adenovirus H5.010RSVTK: a phase 1 trial (BB-IND 6274). *Hum Gene Ther 1996*; **7:** 2047-2057.

55 Sterman DH *et al.* Adenovirus-mediated herpes simplex virus thymidine kinase/ganciclovir gene therapy in patients with localized malignancy: results of a phase I clinical trial in malignant mesothelioma. *Hum Gene Ther 1998*; **9:** 1083-1092.

56 Crystal RG *et al.* Phase I study of direct administration of a replication deficient adenovirus vector containing the E. coli cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. *Hum Gene Ther 1997*; **8:** 985-1001.

57 Freytag SO *et al.* Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res 2002*; **62:** 4968-4976.

58 Palmer DH *et al.* Virus-directed enzyme prodrug therapy: intratumoral administration of a replication-deficient adenovirus encoding nitroreductase to patients with resectable liver cancer. *J Clin Oncol 2004*; **22:** 1546-1552.

59 Blackburn RV, Galoforo SS, Corry PM, Lee YJ. Adenoviral-mediated transfer of a heat-inducible double suicide gene into prostate carcinoma cells. *Cancer Res 1998*; **58:** 1358-1362.

60 Uckert W *et al.* Double suicide gene (cytosine deaminase and herpes simplex virus thymidine kinase) but not single gene transfer allows reliable elimination of tumor cells in vivo. *Hum Gene Ther 1998*; **9:** 855-865.

61 Rogulski KR, Kim JH, Kim SH, Freytag SO. Glioma cells transduced with an Escherichia coli CD/HSV-1 TK fusion gene exhibit enhanced metabolic suicide and radiosensitivity. *Hum Gene Ther 1997*; **8:** 73-85.

62 Aghi M *et al.* Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. *J Natl Cancer Inst 1998*; **90:** 370-380.

63 Rogulski KR *et al.* Pronounced antitumor effects and tumor radiosensitization of double suicide gene therapy. *Clin Cancer Res 1997*; **3:** 2081-2088.

64 Gupta M, Fujimori A, Pommier Y. Eukaryotic DNA topoisomerases I. *Biochim Biophys Acta 1995*; **1262:** 1-14.

65 Liu LF *et al.* Mechanism of action of camptothecin. *Ann N Y Acad Sci 2000*; **922:** 1-10.

66 Shao RG *et al.* Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes. *Embo J 1999*; **18:** 1397-1406.
67 Morris EJ, Geller HM. Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. *J Cell Biol 1996*; **134:** 757-770.
68 Tanizawa A, Fujimori A, Fujimori Y, Pommier Y. Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. *J Natl Cancer Inst 1994*; **86:** 836-842.
69 Houghton JA *et al.* Evaluation of irinotecan in combination with 5-fluorouracil or etoposide in xenograft models of colon adenocarcinoma and rhabdomyosarcoma. *Clin Cancer Res 1996*; **2:** 107-118.
70 Houghton PJ *et al.* Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemother Pharmacol 1995*; **36:** 393-403.
71 Bissery MC, Vrignaud P, Lavelle F, Chabot GG. Experimental antitumor activity and pharmacokinetics of the camptothecin analog irinotecan (CPT-11) in mice. *Anticancer Drugs 1996*; **7:** 437-460.
72 Thompson J *et al.* Efficacy of systemic administration of irinotecan against neuroblastoma xenografts. *Clin Cancer Res 1997*; **3:** 423-431.
73 Thompson J *et al.* Efficacy of oral irinotecan against neuroblastoma xenografts. *Anticancer Drugs 1997*; **8:** 313-322.
74 Furman WL *et al.* Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. *J Clin Oncol 1999*; **17:** 1815-1824.
75 Cunningham D *et al.* Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. *Lancet 1998*; **352:** 1413-1418.
76 Saltz LB *et al.* Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med 2000*; **343:** 905-914.
77 Douillard JY *et al.* Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet 2000*; **355:** 1041-1047.
78 Ilson DH *et al.* Phase II trial of weekly irinotecan plus cisplatin in advanced esophageal cancer. *J Clin Oncol 1999*; **17:** 3270-3275.
79 Hecht JR. Gastrointestinal toxicity or irinotecan. *Oncology (Huntingt) 1998*; **12:** 72-78.
80 Satoh T *et al.* Metabolic activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. *Biol Pharm Bull 1994*; **17:** 662-664.
81 Tsuji T *et al.* CPT-11 converting enzyme from rat serum: purification and some properties. *J Pharmacobiodyn 1991*; **14:** 341-349.
82 Humerickhouse R *et al.* Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res 2000*; **60:** 1189-1192.
83 Dodds HM *et al.* Identification of a new metabolite of CPT-11 (irinotecan): pharmacological properties and activation to SN-38. *J Pharmacol Exp Ther 1998*; **286:** 578-583.
84 Haaz MC, Riche C, Rivory LP, Robert J. Biosynthesis of an aminopiperidino metabolite of irinotecan [7-ethyl-10-[4-(1-piperidino)-1-

piperidino]carbonyloxycamptothecine] by human hepatic microsomes. *Drug Metab Dispos 1998*; **26:** 769-774.
85 Santos A *et al.* Metabolism of irinotecan (CPT-11) by CYP3A4 and CYP3A5 in humans. *Clin Cancer Res 2000*; **6:** 2012-2020.
86 Hanioka N *et al.* Human liver UDP-glucuronosyltransferase isoforms involved in the glucuronidation of 7-ethyl-10-hydroxycamptothecin. *Xenobiotica 2001*; **31:** 687-699.
87 Rivory LP *et al.* Identification and properties of a major plasma metabolite of irinotecan (CPT-11) isolated from the plasma of patients. *Cancer Res 1996*; **56:** 3689-3694.
88 Morton CL, Wadkins RM, Danks MK, Potter PM. The anticancer prodrug CPT-11 is a potent inhibitor of acetylcholinesterase but is rapidly catalyzed to SN-38 by butyrylcholinesterase. *Cancer Res 1999*; **59:** 1458-1463.
89 Rivory LP, Bowles MR, Robert J, Pond SM. Conversion of irinotecan (CPT-11) to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by human liver carboxylesterase. *Biochem Pharmacol 1996*; **52:** 1103-1111.
90 Rivory LP *et al.* Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. *Clin Cancer Res 1997*; **3:** 1261-1266.
91 Hosokawa M, Maki T, Satoh T. Characterization of molecular species of liver microsomal carboxylesterases of several animal species and humans. *Arch Biochem Biophys 1990*; **277:** 219-227.
92 Yamada T *et al.* Immunohistochemistry with an antibody to human liver carboxylesterase in human brain tissues. *Brain Res 1994*; **658:** 163-167.
93 McCracken NW, Blain PG, Williams FM. Human xenobiotic metabolizing esterases in liver and blood. *Biochem Pharmacol 1993*; **46:** 1125-1129.
94 Inoue M *et al.* Hydrolysis of ester-type drugs by the purified esterase from human intestinal mucosa. *Jpn J Pharmacol 1979*; **29:** 17-25.
95 Lund-Pero M *et al.* Non-specific steroidal esterase activity and distribution in human and other mammalian tissues. *Clin Chim Acta 1994*; **224:** 9-20.
96 Hojring N, Svensmark O. Carboxylesterases with defferent substrate specificity in human brain extracts. *J Neurochem 1976*; **27:** 525-528.
97 Munger JS *et al.* A serine esterase released by human alveolar macrophages is closely related to liver microsomal carboxylesterases. *J Biol Chem 1991*; **266:** 18832-18838.
98 Saboori AM, Newcombe DS. Human monocyte carboxylesterase. Purification and kinetics. *J Biol Chem 1990*; **265:** 19792-19799.
99 Satoh T, Hosokawa M. The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol 1998*; **38:** 257-288.
100 Tang BK, Kalow W. Variable activation of lovastatin by hydrolytic enzymes in human plasma and liver. 4. *Eur J Clin Pharmacol 1995*; **47:** 449-451.
101 Woodward DF *et al.* Studies on the ocular hypotensive effects of prostaglandin F2 alpha ester prodrugs and receptor selective prostaglandin analogs. *J Ocul Pharmacol 1994*; **10:** 177-193.
102 Nakamura M, Shirasawa E, Hikida M. Characterization of esterases involved in the hydrolysis of dipivefrin hydrochloride. *Ophthalmic Res 1993*; **25:** 46-51.
103 Huang TL *et al.* Hydrolysis of carbonates, thiocarbonates, carbamates, and carboxylic esters of alpha-naphthol, beta-naphthol, and p-nitrophenol by human, rat, and mouse liver carboxylesterases. *Pharm Res 1993*; **10:** 639-648.

104 Dean RA, Zhang J, Brzezinski MR, Bosron WF. Tissue distribution of cocaine methyl esterase and ethyl transferase activities: correlation with carboxylesterase protein. *J Pharmacol Exp Ther 1995*; **275:** 965-971.
105 Nambu K *et al.* Enzymatic hydrolysis of haloperidol decanoate and its inhibition by proteins. *Biochem Pharmacol 1987*; **36:** 1715-1722.
106 Satoh T *et al.* Current progress on esterases: from molecular structure to function. *Drug Metab Dispos 2002*; **30:** 488-493.
107 Potter PM *et al.* Cellular localization domains of a rabbit and a human carboxylesterase: influence on irinotecan (CPT-11) metabolism by the rabbit enzyme. *Cancer Res 1998*; **58:** 3627-3632.
108 Wadkins RM *et al.* Structural constraints affect the metabolism of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) by carboxylesterases. *Mol Pharmacol 2001*; **60:** 355-362.
109 Potter PM *et al.* Isolation and partial characterization of a cDNA encoding a rabbit liver carboxylesterase that activates the prodrug irinotecan (CPT-11). *Cancer Res 1998*; **58:** 2646-2651.
110 Potter PM *et al.* In situ subcellular localization of epitope-tagged human and rabbit carboxylesterases. *Cytometry 1998*; **32:** 223-232.
111 Danks MK, Morton CL, Pawlik CA, Potter PM. Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. *Cancer Res 1998*; **58:** 20-22.
112 Danks MK *et al.* Comparison of activation of CPT-11 by rabbit and human carboxylesterases for use in enzyme/prodrug therapy. *Clin Cancer Res 1999*; **5:** 917-924.
113 Kojima A, Hackett NR, Crystal RG. Reversal of CPT-11 resistance of lung cancer cells by adenovirus-mediated gene transfer of the human carboxylesterase cDNA. *Cancer Res 1998*; **58:** 4368-4374.
114 Kojima A, Hackett NR, Ohwada A, Crystal RG. In vivo human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors. *J Clin Invest 1998*; **101:** 1789-1796.
115 Wierdl M *et al.* Sensitization of human tumor cells to CPT-11 via adenoviral-mediated delivery of a rabbit liver carboxylesterase. *Cancer Res 2001*; **61:** 5078-5082.
116 Perez MS, Walker LE. Isolation and characterization of a cDNA encoding the KS1/4 epithelial carcinoma marker. *J Immunol 1989*; **142:** 3662-3667.
117 Strnad J *et al.* Molecular cloning and characterization of a human adenocarcinoma/epithelial cell surface antigen complementary DNA. *Cancer Res 1989*; **49:** 314-317.
118 Simon B *et al.* Epithelial glycoprotein is a member of a family of epithelial cell surface antigens homologous to nidogen, a matrix adhesion protein. *Proc Natl Acad Sci U S A 1990*; **87:** 2755-2759.
119 Szala S *et al.* Molecular cloning of cDNA for the carcinoma-associated antigen GA733-2. *Proc Natl Acad Sci U S A 1990*; **87:** 3542-3546.
120 Balzar M *et al.* Cytoplasmic tail regulates the intercellular adhesion function of the epithelial cell adhesion molecule. *Mol Cell Biol 1998*; **18:** 4833-4843.
121 Litvinov SV *et al.* Evidence for a role of the epithelial glycoprotein 40 (Ep-CAM) in epithelial cell-cell adhesion. *Cell Adhes Commun 1994*; **2:** 417-428.
122 Litvinov SV *et al.* Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J Cell Biol 1994*; **125:** 437-446.

123 Litvinov SV *et al.* Epithelial cell adhesion molecule (Ep-CAM) modulates cell-cell interactions mediated by classic cadherins. *J Cell Biol 1997*; **139:** 1337-1348.
124 Winter MJ, Nagtegaal ID, van Krieken JH, Litvinov SV. The epithelial cell adhesion molecule (Ep-CAM) as a morphoregulatory molecule is a tool in surgical pathology. *Am J Pathol 2003*; **163:** 2139-2148.
125 Salem RR *et al.* Expression of colorectal carcinoma-associated antigens in colonic polyps. *J Surg Res 1993*; **55:** 249-255.
126 Packeisen J, Kaup-Franzen C, Knieriem HJ. Detection of surface antigen 17-1A in breast and colorectal cancer. *Hybridoma 1999*; **18:** 37-40.
127 Momburg F, Moldenhauer G, Hammerling GJ, Moller P. Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. *Cancer Res 1987*; **47:** 2883-2891.
128 Takes RP *et al.* Expression of genetic markers in lymph node metastases compared with their primary tumours in head and neck cancer. *J Pathol 2001*; **194:** 298-302.
129 Chaubal S, Wollenberg B, Kastenbauer E, Zeidler R. Ep-CAM--a marker for the detection of disseminated tumor cells in patients suffering from SCCHN. *Anticancer Res 1999*; **19:** 2237-2242.
130 Chang F, Syrjanen S, Syrjanen K. Implications of the p53 tumor-suppressor gene in clinical oncology. *J Clin Oncol 1995*; **13:** 1009-1022.
131 Staib L *et al.* Immunogenicity of recombinant GA733-2E antigen (CO17-1A, EGP, KS1-4, KSA, Ep-CAM) in gastro-intestinal carcinoma patients. *Int J Cancer 2001*; **92:** 79-87.
132 Haller DG. Update of clinical trials with edrecolomab: a monoclonal antibody therapy for colorectal cancer. *Semin Oncol 2001*; **28:** 25-30.
133 Riethmuller G *et al.* Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol 1998*; **16:** 1788-1794.
134 Haisma HJ *et al.* Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen EpCAM. *Gene Ther 1999*; **6:** 1469-1474.
135 Haisma HJ *et al.* Targeting of adenoviral vectors through a bispecific single-chain antibody. *Cancer Gene Ther 2000*; **7:** 901-904.
136 Heideman DA *et al.* Selective gene delivery toward gastric and esophageal adenocarcinoma cells via EpCAM-targeted adenoviral vectors. *Cancer Gene Ther 2001*; **8:** 342-351.
137 Rowe WP *et al.* Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med 1953*; **84:** 570-573.
138 Ginsberg HS, Pereira HG, Valentine RC, Wilcox WC. A proposed terminology for the adenovirus antigens and virion morphological subunits. *Virology 1966*; **28:** 782-783.
139 Chroboczek J, Bieber F, Jacrot B. The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. *Virology 1992*; **186:** 280-285.
140 Robinson AJ, Younghusband HB, Bellett AJ. A circula DNA-protein complex from adenoviruses. *Virology 1973*; **56:** 54-69.
141 Jones N, Shenk T. An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc Natl Acad Sci U S A 1979*; **76:** 3665-3669.

142 Grand RJ. The structure and functions of the adenovirus early region 1 proteins. *Biochem J 1987*; **241:** 25-38.
143 Ginsberg HS *et al.* Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc Natl Acad Sci U S A 1989*; **86:** 3823-3827.
144 Tollefson AE *et al.* The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants. *Virology 1996*; **220:** 152-162.
145 Wold WS. Adenovirus genes that modulate the sensitivity of virus-infected cells to lysis by TNF. *J Cell Biochem 1993*; **53:** 329-335.
146 Bergelson JM *et al.* Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science 1997*; **275:** 1320-1323.
147 Dyson N, Harlow E. Adenovirus E1A targets key regulators of cell proliferation. *Cancer Surv 1992*; **12:** 161-195.
148 Rao L *et al.* The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc Natl Acad Sci U S A 1992*; **89:** 7742-7746.
149 Debbas M, White E. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev 1993*; **7:** 546-554.
150 Yew PR, Liu X, Berk AJ. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. *Genes Dev 1994*; **8:** 190-202.
151 Sarnow P *et al.* Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J Virol 1984*; **49:** 692-700.
152 Sarnow P, Ho YS, Williams J, Levine AJ. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell 1982*; **28:** 387-394.
153 Yew PR, Berk AJ. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature 1992*; **357:** 82-85.
154 Bischoff JR *et al.* An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science 1996*; **274:** 373-376.
155 Stott FJ *et al.* The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *Embo J 1998*; **17:** 5001-5014.
156 Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell 1998*; **92:** 725-734.
157 Scheffner M *et al.* The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell 1990*; **63:** 1129-1136.
158 Heise C *et al.* ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med 1997*; **3:** 639-645.
159 Goodrum FD, Ornelles DA. p53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. *J Virol 1998*; **72:** 9479-9490.
160 Rothmann T *et al.* Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J Virol 1998*; **72:** 9470-9478.
161 Ries SJ *et al.* Loss of p14ARF in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). *Nat Med 2000*; **6:** 1128-1133.
162 Yang CT *et al.* p14(ARF) modulates the cytolytic effect of ONYX-015 in mesothelioma cells with wild-type p53. *Cancer Res 2001*; **61:** 5959-5963.

163 Geoerger B *et al.* Oncolytic activity of the E1B-55 kDa-deleted adenovirus ONYX-015 is independent of cellular p53 status in human malignant glioma xenografts. *Cancer Res 2002*; **62:** 764-772.
164 Hay JG *et al.* Targeting the replication of adenoviral gene therapy vectors to lung cancer cells: the importance of the adenoviral E1b-55kD gene. *Hum Gene Ther 1999*; **10:** 579-590.
165 Hall AR, Dix BR, O'Carroll SJ, Braithwaite AW. p53-dependent cell death/apoptosis is required for a productive adenovirus infection. *Nat Med 1998*; **4:** 1068-1072.
166 Dix BR *et al.* Efficient induction of cell death by adenoviruses requires binding of E1B55k and p53. *Cancer Res 2000*; **60:** 2666-2672.
167 Shen Y *et al.* Analyses of single-amino-acid substitution mutants of adenovirus type 5 E1B-55K protein. *J Virol 2001*; **75:** 4297-4307.
168 Ramachandra M *et al.* Re-engineering adenovirus regulatory pathways to enhance oncolytic specificity and efficacy. *Nat Biotechnol 2001*; **19:** 1035-1041.
169 Helt AM, Galloway DA. Mechanisms by which DNA tumor virus oncoproteins target the Rb family of pocket proteins. *Carcinogenesis 2003*; **24:** 159-169.
170 Fueyo J *et al.* A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene 2000*; **19:** 2-12.
171 Heise C *et al.* An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat Med 2000*; **6:** 1134-1139.
172 Johnson L *et al.* Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. *Cancer Cell 2002*; **1:** 325-337.
173 Egan C *et al.* Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. *Mol Cell Biol 1988*; **8:** 3955-3959.
174 Howe JA *et al.* Evaluation of E1-mutant adenoviruses as conditionally replicating agents for cancer therapy. *Mol Ther 2000*; **2:** 485-495.
175 Balague C *et al.* Human papillomavirus E6E7-mediated adenovirus cell killing: selectivity of mutant adenovirus replication in organotypic cultures of human keratinocytes. *J Virol 2001*; **75:** 7602-7611.
176 Cascallo M, Capella G, Mazo A, Alemany R. Ras-dependent oncolysis with an adenovirus VAI mutant. *Cancer Res 2003*; **63:** 5544-5550.
177 Mundschau LJ, Faller DV. Oncogenic ras induces an inhibitor of double-stranded RNA-dependent eukaryotic initiation factor 2 alpha-kinase activation. *J Biol Chem 1992*; **267:** 23092-23098.
178 Fukuda K *et al.* E1A, E1B double-restricted adenovirus for oncolytic gene therapy of gallbladder cancer. *Cancer Res 2003*; **63:** 4434-4440.
179 Rodriguez R *et al.* Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res 1997*; **57:** 2559-2563.
180 Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res 1999*; **59:** 1498-1504.
181 Yu DC *et al.* The addition of adenovirus type 5 region E3 enables calydon virus 787 to eliminate distant prostate tumor xenografts. *Cancer Res 1999*; **59:** 4200-4203.
182 Matsubara S *et al.* A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res 2001*; **61:** 6012-6019.

183 Bevilacqua A, Ceriani MC, Capaccioli S, Nicolin A. Post-transcriptional regulation of gene expression by degradation of messenger RNAs. *J Cell Physiol 2003*; **195:** 356-372.
184 Hallenbeck PL *et al.* A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther 1999*; **10:** 1721-1733.
185 Takahashi M *et al.* E1B-55K-deleted adenovirus expressing E1A-13S by AFP-enhancer/promoter is capable of highly specific replication in AFP-producing hepatocellular carcinoma and eradication of established tumor. *Mol Ther 2002*; **5:** 627-634.
186 Kurihara T, Brough DE, Kovesdi I, Kufe DW. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. *J Clin Invest 2000*; **106:** 763-771.
187 Hernandez-Alcoceba R, Pihalja M, Wicha MS, Clarke MF. A novel, conditionally replicative adenovirus for the treatment of breast cancer that allows controlled replication of E1a-deleted adenoviral vectors. *Hum Gene Ther 2000*; **11:** 2009-2024.
188 Zhang L *et al.* Adenoviral vectors with E1A regulated by tumor-specific promoters are selectively cytolytic for breast cancer and melanoma. *Mol Ther 2002*; **6:** 386-393.
189 Nettelbeck DM *et al.* Novel oncolytic adenoviruses targeted to melanoma: specific viral replication and cytolysis by expression of E1A mutants from the tyrosinase enhancer/promoter. *Cancer Res 2002*; **62:** 4663-4670.
190 Peter I *et al.* A novel attenuated replication-competent adenovirus for melanoma therapy. *Gene Ther 2003*; **10:** 530-539.
191 Hamada K *et al.* Identification of the human IAI.3B promoter element and its use in the construction of a replication-selective adenovirus for ovarian cancer therapy. *Cancer Res 2003*; **63:** 2506-2512.
192 Doronin K *et al.* Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. *J Virol 2001*; **75:** 3314-3324.
193 Brunori M, Malerba M, Kashiwazaki H, Iggo R. Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway. *J Virol 2001*; **75:** 2857-2865.
194 Fuerer C, Iggo R. Adenoviruses with Tcf binding sites in multiple early promoters show enhanced selectivity for tumour cells with constitutive activation of the wnt signalling pathway. *Gene Ther 2002*; **9:** 270-281.
195 Hernandez-Alcoceba R, Pihalja M, Qian D, Clarke MF. New oncolytic adenoviruses with hypoxia- and estrogen receptor- regulated replication. *Hum Gene Ther 2002*; **13:** 1737-1750.
196 Cuevas Y *et al.* Specific oncolytic effect of a new hypoxia-inducible factor-dependent replicative adenovirus on von Hippel-Lindau-defective renal cell carcinomas. *Cancer Res 2003*; **63:** 6877-6884.
197 Tsukuda K *et al.* An E2F-responsive replication-selective adenovirus targeted to the defective cell cycle in cancer cells: potent antitumoral efficacy but no toxicity to normal cell. *Cancer Res 2002*; **62:** 3438-3447.
198 Jakubczak JL *et al.* An oncolytic adenovirus selective for retinoblastoma tumor suppressor protein pathway-defective tumors: dependence on E1A, the E2F-1 promoter, and viral replication for selectivity and efficacy. *Cancer Res 2003*; **63:** 1490-1499.

199 Savontaus MJ, Sauter BV, Huang TG, Woo SL. Transcriptional targeting of conditionally replicating adenovirus to dividing endothelial cells. *Gene Ther 2002*; **9:** 972-979.
200 Ring CJ, Harris JD, Hurst HC, Lemoine NR. Suicide gene expression induced in tumour cells transduced with recombinant adenoviral, retroviral and plasmid vectors containing the ERBB2 promoter. *Gene Ther 1996*; **3:** 1094-1103.
201 Vassaux G, Hurst HC, Lemoine NR. Insulation of a conditionally expressed transgene in an adenoviral vector. *Gene Ther 1999*; **6:** 1192-1197.
202 Steinwaerder DS, Lieber A. Insulation from viral transcriptional regulatory elements improves inducible transgene expression from adenovirus vectors in vitro and in vivo. *Gene Ther 2000*; **7:** 556-567.
203 Ahmed A *et al.* A conditionally replicating adenovirus targeted to tumor cells through activated RAS/P-MAPK-selective mRNA stabilization. *Nat Biotechnol 2003*; **21:** 771-777.
204 Derrigo M, Cestelli A, Savettieri G, Di Liegro I. RNA-protein interactions in the control of stability and localization of messenger RNA (review). *Int J Mol Med 2000*; **5:** 111-123.
205 Ross J. mRNA stability in mammalian cells. *Microbiol Rev 1995*; **59:** 423-450.
206 Nemunaitis J *et al.* Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. *Cancer Res 2000*; **60:** 6359-6366.
207 Mulvihill S *et al.* Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. *Gene Ther 2001*; **8:** 308-315.
208 Habib N *et al.* Clinical trial of E1B-deleted adenovirus (dl1520) gene therapy for hepatocellular carcinoma. *Cancer Gene Ther 2002*; **9:** 254-259.
209 Ganly I *et al.* A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res 2000*; **6:** 798-806.
210 Makower D *et al.* Phase II clinical trial of intralesional administration of the oncolytic adenovirus ONYX-015 in patients with hepatobiliary tumors with correlative p53 studies. *Clin Cancer Res 2003*; **9:** 693-702.
211 Nemunaitis J *et al.* Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol 2001*; **19:** 289-298.
212 Vasey PA *et al.* Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J Clin Oncol 2002*; **20:** 1562-1569.
213 Habib NA *et al.* E1B-deleted adenovirus (dl1520) gene therapy for patients with primary and secondary liver tumors. *Hum Gene Ther 2001*; **12:** 219-226.
214 Reid T *et al.* Intra-arterial administration of a replication-selective adenovirus (dl1520) in patients with colorectal carcinoma metastatic to the liver: a phase I trial. *Gene Ther 2001*; **8:** 1618-1626.
215 Nemunaitis J *et al.* Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. *Gene Ther 2001*; **8:** 746-759.

216 DeWeese TL *et al.* A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res 2001*; **61:** 7464-7472.
217 Heise C, Lemmon M, Kirn D. Efficacy with a replication-selective adenovirus plus cisplatin-based chemotherapy: dependence on sequencing but not p53 functional status or route of administration. *Clin Cancer Res 2000*; **6:** 4908-4914.
218 You L, Yang CT, Jablons DM. ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res 2000*; **60:** 1009-1013.
219 Sanchez-Prieto R *et al.* Carcinoma cell lines become sensitive to DNA-damaging agents by the expression of the adenovirus E1A gene. *Oncogene 1996*; **13:** 1083-1092.
220 Sparer TE *et al.* The role of human adenovirus early region 3 proteins (gp19K, 10.4K, 14.5K, and 14.7K) in a murine pneumonia model. *J Virol 1996*; **70:** 2431-2439.
221 Li Y *et al.* A hepatocellular carcinoma-specific adenovirus variant, CV890, eliminates distant human liver tumors in combination with doxorubicin. *Cancer Res 2001*; **61:** 6428-6436.
222 Yu DC *et al.* Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res 2001*; **61:** 517-525.
223 Hemminki A *et al.* Modulation of coxsackie-adenovirus receptor expression for increased adenoviral transgene expression. *Cancer Res 2003*; **63:** 847-853.
224 Khuri FR *et al.* a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med 2000*; **6:** 879-885.
225 Hecht JR *et al.* A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. *Clin Cancer Res 2003*; **9:** 555-561.
226 Rogulski KR *et al.* In vivo antitumor activity of ONYX-015 is influenced by p53 status and is augmented by radiotherapy. *Cancer Res 2000*; **60:** 1193-1196.
227 Geoerger B *et al.* Potentiation of radiation therapy by the oncolytic adenovirus dl1520 (ONYX-015) in human malignant glioma xenografts. *Br J Cancer 2003*; **89:** 577-584.
228 Toth K *et al.* Radiation increases the activity of oncolytic adenovirus cancer gene therapy vectors that overexpress the ADP (E3-11.6K) protein. *Cancer Gene Ther 2003*; **10:** 193-200.
229 Zhang M *et al.* Ionizing radiation increases adenovirus uptake and improves transgene expression in intrahepatic colon cancer xenografts. *Mol Ther 2003*; **8:** 21-28.
230 Chen Y *et al.* CV706, a prostate cancer-specific adenovirus variant, in combination with radiotherapy produces synergistic antitumor efficacy without increasing toxicity. *Cancer Res 2001*; **61:** 5453-5460.
231 Lamfers ML *et al.* Potential of the conditionally replicative adenovirus Ad5-Delta24RGD in the treatment of malignant gliomas and its enhanced effect with radiotherapy. *Cancer Res 2002*; **62:** 5736-5742.
232 Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci U S A 1997*; **94:** 3352-3356.

233 Miller CR *et al.* Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res 1998*; **58:** 5738-5748.
234 Grill J *et al.* Combined targeting of adenoviruses to integrins and epidermal growth factor receptors increases gene transfer into primary glioma cells and spheroids. *Clin Cancer Res 2001*; **7:** 641-650.
235 Witlox MA *et al.* Epidermal growth factor receptor targeting enhances adenoviral vector based suicide gene therapy of osteosarcoma. *J Gene Med 2002*; **4:** 510-516.
236 Wesseling JG *et al.* Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors. *Gene Ther 2001*; **8:** 969-976.
237 Li D, Duan L, Freimuth P, O'Malley BW, Jr. Variability of adenovirus receptor density influences gene transfer efficiency and therapeutic response in head and neck cancer. *Clin Cancer Res 1999*; **5:** 4175-4181.
238 Cripe TP *et al.* Fiber knob modifications overcome low, heterogeneous expression of the coxsackievirus-adenovirus receptor that limits adenovirus gene transfer and oncolysis for human rhabdomyosarcoma cells. *Cancer Res 2001*; **61:** 2953-2960.
239 Douglas JT *et al.* Efficient oncolysis by a replicating adenovirus (ad) in vivo is critically dependent on tumor expression of primary ad receptors. *Cancer Res 2001*; **61:** 813-817.
240 Defer C, Belin MT, Caillet-Boudin ML, Boulanger P. Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. *J Virol 1990*; **64:** 3661-3673.
241 Stevenson SC *et al.* Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. *J Virol 1995*; **69:** 2850-2857.
242 Krasnykh VN, Mikheeva GV, Douglas JT, Curiel DT. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J Virol 1996*; **70:** 6839-6846.
243 Stevenson SC, Rollence M, Marshall-Neff J, McClelland A. Selective targeting of human cells by a chimeric adenovirus vector containing a modified fiber protein. *J Virol 1997*; **71:** 4782-4790.
244 Kanerva A *et al.* Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. *Clin Cancer Res 2002*; **8:** 275-280.
245 Von Seggern DJ *et al.* Adenovirus vector pseudotyping in fiber-expressing cell lines: improved transduction of Epstein-Barr virus-transformed B cells. *J Virol 2000*; **74:** 354-362.
246 Haviv YS *et al.* Adenoviral gene therapy for renal cancer requires retargeting to alternative cellular receptors. *Cancer Res 2002*; **62:** 4273-4281.
247 Kawakami Y *et al.* Substitution of the adenovirus serotype 5 knob with a serotype 3 knob enhances multiple steps in virus replication. *Cancer Res 2003*; **63:** 1262-1269.
248 Kanerva A *et al.* Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus. *Mol Ther 2003*; **8:** 449-458.
249 Krasnykh VN, Douglas JT, van Beusechem VW. Genetic targeting of adenoviral vectors. *Mol Ther 2000*; **1:** 391-405.
250 Roelvink PW *et al.* Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science 1999*; **286:** 1568-1571.

251 Shinoura N *et al.* Highly augmented cytopathic effect of a fiber-mutant E1B-defective adenovirus for gene therapy of gliomas. *Cancer Res 1999*; **59:** 3411-3416.
252 Suzuki K *et al.* A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic potency. *Clin Cancer Res 2001*; **7:** 120-126.
253 Bauerschmitz GJ *et al.* Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. *Cancer Res 2002*; **62:** 1266-1270.
254 Fueyo J *et al.* Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. *J Natl Cancer Inst 2003*; **95:** 652-660.
255 Tollefson AE *et al.* The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol 1996*; **70:** 2296-2306.
256 Suzuki K, Alemany R, Yamamoto M, Curiel DT. The presence of the adenovirus E3 region improves the oncolytic potency of conditionally replicative adenoviruses. *Clin Cancer Res 2002*; **8:** 3348-3359.
257 Douglas JT *et al.* Targeted gene delivery by tropism-modified adenoviral vectors. *Nat Biotechnol 1996*; **14:** 1574-1578.
258 Doukas J *et al.* Retargeted delivery of adenoviral vectors through fibroblast growth factor receptors involves unique cellular pathways. *Faseb J 1999*; **13:** 1459-1466.
259 van der Poel HG *et al.* Epidermal growth factor receptor targeting of replication competent adenovirus enhances cytotoxicity in bladder cancer. *J Urol 2002*; **168:** 266-272.
260 Hemminki A *et al.* Targeting oncolytic adenoviral agents to the epidermal growth factor pathway with a secretory fusion molecule. *Cancer Res 2001*; **61:** 6377-6381.
261 Hemminki A *et al.* Production of an EGFR targeting molecule from a conditionally replicating adenovirus impairs its oncolytic potential. *Cancer Gene Ther 2003*; **10:** 583-588.
262 Van Beusechem VW *et al.* Conditionally replicative adenovirus expressing a targeting adapter molecule exhibits enhanced oncolytic potency on CAR-deficient tumors. *Gene Ther 2003*; **10:** 1982-1991.
263 Einfeld DA *et al.* Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. *J Virol 2001*; **75:** 11284-11291.
264 van Beusechem VW *et al.* Efficient and selective gene transfer into primary human brain tumors by using single-chain antibody-targeted adenoviral vectors with native tropism abolished. *J Virol 2002*; **76:** 2753-2762.
265 Wildner O *et al.* Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. *Gene Ther 1999*; **6:** 57-62.
266 Wildner O, Blaese RM, Morris JC. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. *Cancer Res 1999*; **59:** 410-413.
267 Wildner O, Morris JC. The role of the E1B 55 kDa gene product in oncolytic adenoviral vectors expressing herpes simplex virus-tk: assessment of antitumor efficacy and toxicity. *Cancer Res 2000*; **60:** 4167-4174.
268 Morris JC, Wildner O. Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-tk. *Mol Ther 2000*; **1:** 56-62.
269 Lambright ES *et al.* Inclusion of the herpes simplex thymidine kinase gene in a replicating adenovirus does not augment antitumor efficacy. *Gene Ther 2001*; **8:** 946-953.

270 Nanda D *et al.* Treatment of malignant gliomas with a replicating adenoviral vector expressing herpes simplex virus-thymidine kinase. *Cancer Res 2001*; **61:** 8743-8750.
271 Rogulski KR *et al.* Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. *Hum Gene Ther 2000*; **11:** 67-76.
272 Freytag SO *et al.* A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther 1998*; **9:** 1323-1333.
273 Freytag SO *et al.* Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model. *Int J Radiat Oncol Biol Phys 2002*; **54:** 873-885.
274 Stubdal H *et al.* A prodrug strategy using ONYX-015-based replicating adenoviruses to deliver rabbit carboxylesterase to tumor cells for conversion of CPT-11 to SN-38. *Cancer Res 2003*; **63:** 6900-6908.
275 Doronin K *et al.* Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *J Virol 2000*; **74:** 6147-6155.
276 Mi J *et al.* Induced apoptosis supports spread of adenovirus vectors in tumors. *Hum Gene Ther 2001*; **12:** 1343-1352.
277 van Beusechem VW *et al.* Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer Res 2002*; **62:** 6165-6171.
278 Sauthoff H *et al.* Late expression of p53 from a replicating adenovirus improves tumor cell killing and is more tumor cell specific than expression of the adenoviral death protein. *Hum Gene Ther 2002*; **13:** 1859-1871.
279 Geoerger B *et al.* Expression of p53, or targeting towards EGFR, enhances the oncolytic potency of conditionally replicative adenovirus against neuroblastoma. *J Gene Med 2005*.
280 Zhang JF *et al.* Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy. *Proc Natl Acad Sci U S A 1996*; **93:** 4513-4518.
281 Bristol JA *et al.* In vitro and in vivo activities of an oncolytic adenoviral vector designed to express GM-CSF. *Mol Ther 2003*; **7:** 755-764.
282 Bauzon M *et al.* Multigene expression from a replicating adenovirus using native viral promoters. *Mol Ther 2003*; **7:** 526-534.
283 Bateman AR *et al.* Viral fusogenic membrane glycoproteins kill solid tumor cells by nonapoptotic mechanisms that promote cross presentation of tumor antigens by dendritic cells. *Cancer Res 2002*; **62:** 6566-6578.
284 Li H *et al.* Human immunodeficiency virus type 1-mediated syncytium formation is compatible with adenovirus replication and facilitates efficient dispersion of viral gene products and de novo-synthesized virus particles. *Hum Gene Ther 2001*; **12:** 2155-2165.
285 Ahmed A *et al.* Intratumoral expression of a fusogenic membrane glycoprotein enhances the efficacy of replicating adenovirus therapy. *Gene Ther 2003*; **10:** 1663-1671.
286 Hawkins LK, Hermiston TW. Gene delivery from the E3 region of replicating human adenovirus: evaluation of the ADP region. *Gene Ther 2001*; **8:** 1132-1141.

# Chapter 2

# Secreted and tumor targeted carboxylesterase for activation of irinotecan

Dinja Oosterhoff[1], Herbert M. Pinedo[1], Ida H. van der Meulen[1], Michelle de Graaf[1], Tomomichi Sone[2], Frank A. Kruyt[1], Victor W. van Beusechem[1] , Hidde J. Haisma[3] and Winald R. Gerritsen[1]

Division of Gene Therapy, Department of Medical Oncology , VU University Medical Center, Amsterdam, The Netherlands
[2] Department of Toxicology, Faculty of Pharmaceutical Sciences, Setsunan University, Osaka, Japan
[3] Department of Therapeutic Gene Modulation, University Center for Pharmacy, Groningen, The Netherlands

British Journal of Cancer 2002, 87: 659-665

## SUMMARY

Irinotecan (CPT-11) is an anticancer agent for the treatment of colon cancer. CPT-11 can be considered as a prodrug, since it needs to be activated into the toxic drug SN-38 by the enzyme carboxylesterase (CE). An approach to achieve tumor specific activation of CPT-11 is to transduce the cDNA encoding CE into tumor cells. A secreted form of CE may diffuse through a tumor mass and may activate CPT-11 extracellularly. This could enhance the anti-tumor efficacy by exerting a bystander effect on untransduced cells. In addition a secreted targeted-targeted form of CE should prevent leakage of the enzyme from the site of the targeted into the circulation. We have constructed a secreted form of human liver CE-2 by deletion of the cellular retention signal and by cloning the cDNA downstream of an Ig kappa leader sequence. The protein was secreted by transfected cells and showed both enzyme activity and efficient CPT-11 activation. To obtain a secreted, targeted-targeted form of CE2 the cDNA encoding the human scFv antibody C28 directed against the epithelial cell adhesion molecule EpCAM, was inserted between the leader sequence and CE-2. This fusion protein showed CPT-11 activation and specific binding to EpCAM expressing cells. Importantly, in combination with CPT-11 both recombinant CE proteins exerted strong antiproliferative effects on human colon cancer cells. They are, therefore, promising new tools for gene directed enzyme prodrug therapy approaches for the treatment of colon carcinoma with CPT-11.


## INTRODUCTION

Conventional chemotherapy lacks specificity for tumor cells. This results in dose-limiting side effects and insufficient concentrations of the drugs in the tumor, through which efficacy is limited and drug resistant cellular subpopulations may emerge. These problems may be overcome by expressing an enzyme that is capable of converting a non-toxic prodrug into a toxic drug specifically in tumor cells. This so-called gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy aims to increase the concentration of the drug in the tumor while reducing the systemic toxicity. The gene encoding the prodrug-activating enzyme is delivered to the tumor cells by, for example, an adenoviral vector, followed by systemic administration of the prodrug. In this regard, several prodrug-converting enzymes have been extensively studied, such as the herpes simplex virus thymidine kinase enzyme that converts ganciclovir (GCV) into the active compound GCV-P and bacterial cytosine deaminase that activates 5-FC to the anticancer drug 5-FU [1,2].

A prodrug for the treatment of colon carcinoma is irinotecan (CPT-11 or 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin). CPT-11 is converted by carboxylesterases (CE) into the toxic drug SN-38 (7-ethyl-10-hydroxycamptothecin) by cleavage of the bulky dipiperidino side chain at the carbon position [3,4]. CPT-11 has demonstrated anti-tumor activity in immune deprived animals bearing human tumor xenografts [5-8] and is approved for use in the treatment of metastatic colorectal cancer in humans. Although SN-38 can be detected in the plasma of cancer patients only minutes after the

administration of CPT-11 [9], 90% of the administered CPT-11 is not converted to SN-38 [10].

*CE*s are a ubiquitously expressed class of enzymes. High levels of enzyme activity are found in human liver and lung [11]. Different isoforms of human CE have been described. CE1 is found in liver only, whereas CE2 is also found in the intestines and CE3 is found in brain cells [12,13]. Furthermore, it has been shown that human alveolar macrophages release a serine esterase that is identical to liver CE1 [14].

Several studies have been performed using CPT-11 in combination with human CE1 in a GDEPT approach. Kojima *et al*. described the construction of a replication deficient adenoviral vector containing the human liver *CE1* gene driven by the CMV promoter [15,16]. *In vitro* results showed that several tumor cell lines infected with this virus express *CE1* and in the presence of CPT-11 tumor growth was effectively suppressed. However, on many other tumor cell lines only minimal effects were observed. This underscored the notice that the success of a GDEPT approach for CPT-11 requires an enzyme with a high efficiency of converting CPT-11 to SN-38. The rabbit CE was found to be 100-1000 fold more efficient in converting CPT-11 than human liver CE1 and was 12-55 fold more efficient in sensitizing transfected cells to CPT-11 [17]. Therefore, an adenoviral vector expressing rabbit *CE* was constructed and transduction of human tumor cells led to sensitization to CPT-11 [18]. The disadvantage of rabbit CE, however, is that expression of a nonhuman protein in patients may lead to an immunological response and subsequent enzyme inactivation. A human enzyme with higher affinity and higher efficiency than CE1 may overcome these limitations. It was shown that human CE2 has a higher affinity and a higher conversion velocity for CPT-11 than CE1 [19]. Therefore, we envisaged that CE2 would be a candidate to employ in a GDEPT approach to treat human tumors.

To achieve efficient kill of all tumor cells, a bystander effect is required, whereby CPT-11 is cleaved to SN-38 that not only kills the tumor cells in which CE2 is formed, but also neighboring tumor cells that do not express *CE2*. We hypothesized that extracellular conversion of CPT-11 would lead to a larger bystander effect than intracellular conversion and, furthermore, that a fusion protein consisting of secreted CE2 fused to a tumor specific scFv antibody will be retained in the tumor thereby preventing leakage of the enzyme into the circulation and therefore further reducing unwanted side effects.

In this study we describe the construction of a secreted form of CE2 (sCE2) by deletion of a C-terminal cellular retention signal and by adding the Ig kappa leader sequence. Furthermore, a secreted targeted form of human CE2 (C28-sCE2) was constructed by fusing sCE2 to a human scFv directed against Epithelial Cell Adhesion Molecule (EpCAM). The binding specificity and enzyme activity of the secreted form of CE2 and the fusion protein and their ability to sensitize human tumor cell lines to CPT-11 is determined and compared to wild type intracellularly expressed human *CE2* (CE2).

## MATERIAL AND METHODS

### *Chemicals*

Pwo polymerase, PCR buffer and dNTPs were obtained from Roche (Almere, The Netherlands). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Life Technologies (Breda, The Netherlands). The kits used for DNA isolation, purification and extraction from agarose gel were from Qiagen (Hilden, Germany). The substrate p-nitrophenyl-acetate was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The prodrug CPT-11 and the drug SN-38 were obtained from Rhône-Poulenc Rorer (Vitry-sur-Seine, France).

### *Cell lines*

The COS-7 and the human colon cancer SW1398 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) supplemented with 5% (COS-7) or 10% heat-inactivated fetal calf serum (Life Technologies), 50IU/ml penicillin (Life Technologies) and 50μg/ml streptomycin (Life Technologies) in a humidified atmosphere containing 5% $CO_2$ at 37°C.

### *Construction of psCE2 and pC28-sCE2*

The pBluescript vector containing the *CE2* open reading frame [20] was digested with *EcoRI* and the CE2 encoding fragment was ligated into the *EcoRI* linearized eukaryotic expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands). This construct was called pCE2. To construct a secreted form of CE2 (psCE2), two primers (sense 5' GACGC*GGCCCAGCCGGCC*CAGGACTCAGCCAGTCCCATCC 3' and antisense 5' GACTCGA*GCGGCCGC*TCTCTCTTCAGGCTCCTCGAGC 3') were designed to introduce an *SfiI* restriction site (italic) at the beginning of the sequence encoding the mature protein and a *NotI* restriction site (italic) before the retention signal, as shown in figure 1. After performing the PCR, the *SfiI*/*NotI* digested fragment was isolated, purified, and ligated into the eukaryotic expression vector pSTCF, containing a myc- and 6 his-tag and the Ig kappa leader sequence that directs proteins to the secretory pathway [21].

The human anti-EpCAM scFv C28 was derived from scFv UBS-54, which was isolated from a semi-synthetic phage antibody display library [22], and was a kind gift of Dr. T. Logtenberg (Crucell, Leiden). An *SfiI*/*NotI* fragment encoding the scFv *C28* was isolated from a pHEN vector, and cloned into the eukaryotic expression vector pSTCF. A flexible $(Gly_4Ser)_2$ linker was introduced downstream of C28, as described previously [23]. To allow insertion of *sCE2* downstream of the $(Gly_4Ser)_2$ linker, a PCR was performed to obtain a DNA fragment encoding a secreted form of CE2 starting from the mature CE2 protein and ending just before the cellular retention signal. Both primers used in the PCR (sense 5' GTGT*GCGGCCGC*CAGGACTCAGCCAGTCCCATC 3' and antisense primer as described above) contained a *NotI* site (italic). The PCR product was digested with *NotI* and inserted into the *NotI* sites of the vector containing *C28* with the $(Gly_4Ser)_2$ linker to obtain pC28-sCE2.

***Expression of CE2, sCE2 and C28-sCE2 fusion protein***

COS-7 cells ($2.10^6$) were transfected with 2 μg pCE2, psCE2 or pC28-sCE2 by Lipofectamine Plus reagent (Life Technologies) according to instructions of the manufacturer. Cells were grown in 3.5 ml DMEM containing 5% FCS and antibiotics. After 48 h, supernatants were removed and cells were harvested by trypsinization. Cellular lysates were obtained by three times freeze thawing in 350 μl PBS. For cytotoxicity assays and HPLC analysis proteins present in supernatants of transfected COS-7 cells were 10X concentrated using a Biomax-10 centrifugal filter (Millipore, Bedford, USA). Supernatants and cellular lysates were analyzed for the presence of functional CE enzyme or C28-sCE2 fusion protein by Western blotting, esterase activity assay and cytotoxicity assays. Binding of proteins in supernatants of COS-7 cells transfected with pC28-sCE2 or psCE2 to EpCAM positive cells was determined by FACS analysis.

***Western blot analysis***

Proportional amounts of supernatant or cellular lysate from COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2 were dissolved in sample buffer [24] with 5% 2-mercaptoethanol and heated at 95°C for 5 min. Samples were electrophorezed through a denaturing 10% sodium dodecyl sulphate-polyacrylamide gel and protein bands were electroblotted onto PVDF protein membrane (BioRad). Proteins were detected using anti-myc antibody 9E10 [25] and HRP-conjugated rabbit anti-mouse IgG (Dako) or with rabbit-anti-CE2, an antibody directed to the C-terminal retention signal of CE which was a kind gift of Dr. Yan, University of Rhode Island [26], and HRP-conjugated swine anti-rabbit IgG (Dako). Blots were developed with enhanced chemo luminescence reagent (Lumilight Plus, Roche).

***Esterase activity assay***

Supernatants or cellular lysates of transfected COS-7 cells were incubated with 200 μl 100 mM Tris-HCl pH 8.0 containing 100 mM pNpAc, a substrate for CE. After mixing, conversion to p-Nitrophenol was measured at a wavelength of 415 nm during 10 minutes using an ELISA plate reader (BioRad, Veenendaal, The Netherlands).

***FACS analysis***

EpCAM expressing SW1398 cells were trypsinized for 5 min at 37°C, washed with DMEM, counted and resuspended in PBS. A total of $5\times10^5$ cells was incubated for 1 h on ice with 50 μl supernatant of COS-7 cells transfected with pC28-sCE2. As a negative control, supernatants of untransfected COS-7 cells or cells transfected with psCE2 were used. After washing 3 times with PBS, cells were incubated with anti-myc antibody 9E10 in PBS/0.1% BSA, washed 3 times with PBS, and stained with fluorescein-conjugated rabbit anti-mouse IgG (Dako). As a positive control 50 μl (10 μg/ml) of the anti-EpCAM antibody 323/A3 [27] was used. Stained cells were fixed with 1% formaldehyde in PBS

and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

***Immunohistochemistry***

To show binding of C28-sCE2 to EpCAM expressing cells, $1.10^4$ SW1398 cells were plated and incubated overnight with the concentrated supernatants of COS-7 cells transfected with psCE2 or pC28-sCE2. Unbound enzyme was removed by washing with culture medium and cells were fixed with 100 μl 50% MeOH/50% acetone. After washing with PBS, anti-myc antibody 9E10 was added to the cells for 1 h at 37°C, followed by incubation with rabbit anti mouse HRP (1:100 in PBS/0.1%BSA) for 1 h. Hereafter cells were washed and 3-amino-9-ethylcarbazole substrate chromogen (Dako, USA) was added. The staining was stopped by washing with PBS. Cells were counterstained with haematoxylin.

***In vitro cytotoxicity assay***

SW1398 cells ($1.10^4$) were plated in a 96-wells microtiter plate (Bio-one). After 24 h, concentrated supernatants of COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2 was added together with a non-toxic concentration of CPT-11 (1μM). Control experiments were performed in which SW1398 cells were incubated with DMEM supplemented with 10% FCS, SN-38 or CPT-11 only. After another 72 h culture the cells were incubated with cell proliferation reagent WST-1 (Roche Diagnostics) for 1 h at 37°C. The absorbency was measured at a wavelength of 450 nm. The antiproliferative effects were determined and expressed as percentages of growth as compared to untreated control growth, which was set to 100%.

## RESULTS

***Construction of CE2, sCE2 and C28-sCE2***

The cDNA coding for human CE2 [20] was inserted into the eukaryotic expression vector pcDNA3, creating pCE2 (fig. 1). Using PCR we amplified a *CE2* cDNA fragment encoding the mature protein without the last four amino acids encoding the cellular retention signal HTEL. This fragment was inserted into the pSTCF vector, which contains the Ig kappa leader that directs the protein in the secretory pathway and a myc- and 6xhis-tag [21]. The resulting construct encoding a secreted form of CE2 (sCE2) was designated psCE2 (fig.1). The cDNA fragment coding for sCE2 was also inserted into the pSTCF vector in frame with the anti-EpCAM scFv C28, creating pC28-sCE2 (fig. 1).

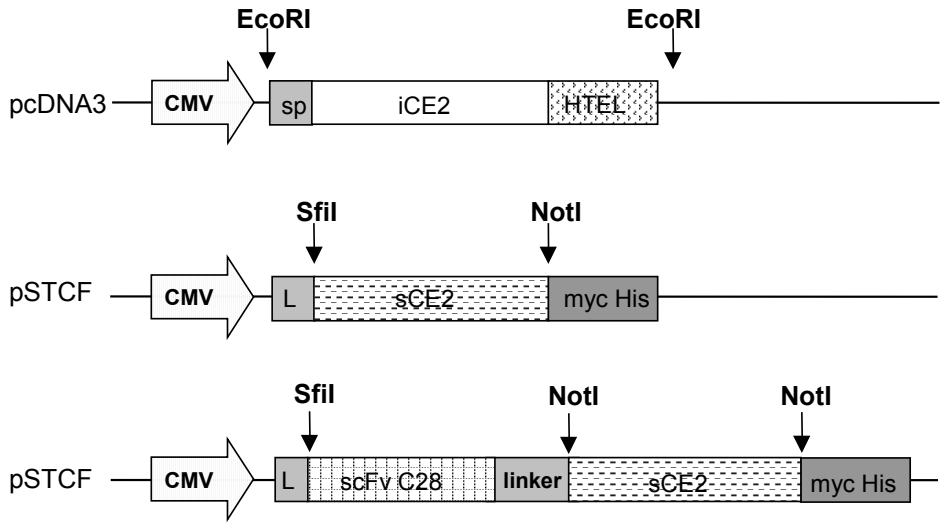



***Figure 1:*** *Schematic representation of the CE2, sCE2 and C28-sCE2 expression cassette. The CE2 cDNA is inserted as an EcoRI fragment into pcDNA3. The encoded protein contains its wild type N-terminal signal peptide and a C-terminal cellular retention signal sequence HTEL. The structural elements of pSTCF include the strong cytomegalovirus (CMV) promoter, IgG kappa leader sequence, and a C-terminal myc- and His-tag (mycHis) for easy detection and purification. sCE2, without retention signal, is inserted as a SfiI/NotI fragment into pSTCF.*

*The anti-EpCAM scFv C28 is inserted as a SfiI/NotI fragment. The gene encoding CE2 is inserted as a NotI/NotI fragment, after the $(Gly_4Ser)_2$ linker is inserted in the NotI and ApaI restriction sites.*

### *Expression and characterization of CE2, sCE2 and C28-sCE2*

COS-7 cells were transfected with pCE2, psCE2 or pC28-sCE2 and expressed proteins in supernatant and cellular lysates were analyzed by Western blotting, FACS analysis, esterase activity assay and cytotoxicity assays. To assess the size of the expressed proteins and determine the amount of secreted protein, SDS-PAGE was performed followed by Western blotting and detection with anti-myc antibody for sCE2 and C28-sCE2 or anti CE2 antibody for CE2 (figure 2). The CE2 protein appeared to remain intracellular since it was only detected in the cellular lysate (fig. 2, lane 1) of transfected COS-7 cells. Like CE2, the sCE2 monomers migrated with an apparent molecular weight of 75 kDa. As expected, the majority of sCE2 was detected in the supernatant of transfected COS-7 cells, proving that deletion of the C-terminal retention signal and fusing the Ig kappa leader, indeed directed the protein into the secretory pathway (fig. 2, lane 5). The C28-sCE2 fusion protein, with an apparent molecular weight of 100kDa, was also found mainly in the supernatants of transfected COS-7 cells (fig. 2, lane 6).

Functional enzyme activity of CE2, sCE2 and C28-sCE2 was demonstrated by an esterase enzyme activity assay (figure 3). In cells transfected with pCE2 esterase activity remained intracellular, while in cells transfected with psCE2 or pC28-sCE2 almost all activity was detected in the culture medium. These results confirmed the results of the Western blotting

experiments, since the relative amounts and the activities of CE2 proteins in cells and supernatants of transfected COS-7 cells were comparable.

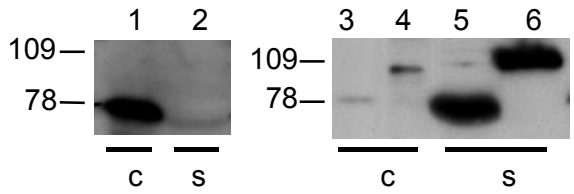



***Figure 2:*** *Western blot analysis of the cellular lysates and supernatants of COS-7 cells transfected with pCE, psCE2 or pC28-sCE2. sCE2 and C28-sCE2 were detected using an antibody directed against the myc-tag and CE2 was detected with an antibody directed against the C-terminal cellular retention signal. In lanes 1, 3 and 4 cellular lysates (c) and in lanes 2, 5 and 6 supernatants (s) of COS-7 cells transfected with pCE2 (lanes 1,2), psCE2 (lanes 3,4) and pC28-sCE2 (lanes 5,6) respectively are shown. The CE proteins migrated with an apparent molecular weight of 75 kDa whereas the fusion protein had a molecular weight of 100 kDa. As expected, CE2 mainly remained intracellular while sCE2 and C28-sCE2 were secreted by transfected COS-7 cells.*

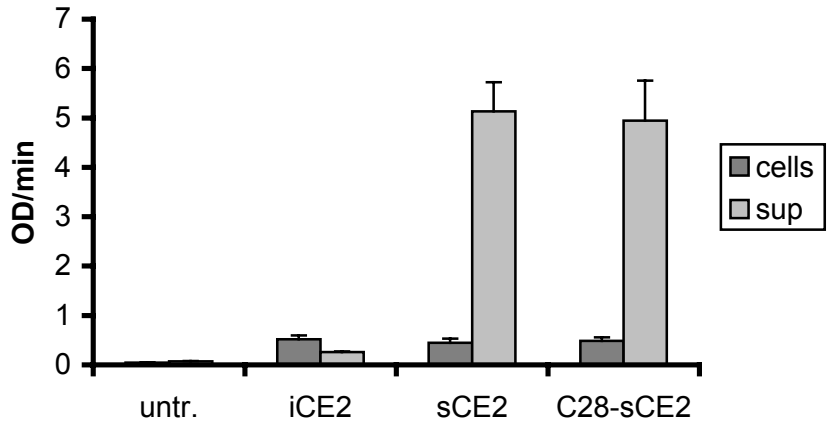
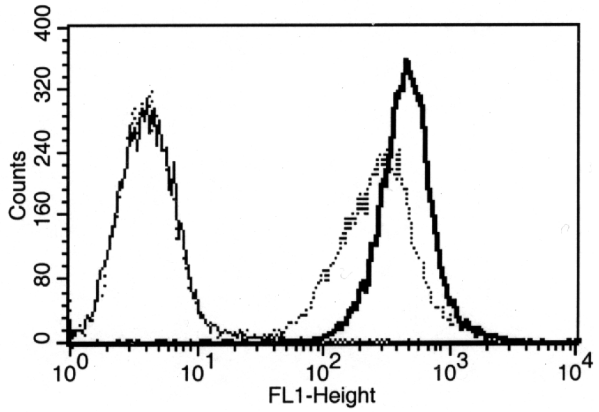



***Figure 3:*** *CE-activity in cellular lysates and supernatants of COS-7 transfected with pCE2, psCE2, pC28-sCE2. Cellular lysates or supernatants of transfected COS-7 cells were incubated with 1mM pNpAc and conversion was measured during 10 minutes. sCE2 and C28-sCE2 show enzymatic activity and are efficiently secreted by transfected cells, because most of the enzyme activity is found in the supernatant.*

Binding of the C28-sCE2 fusion protein to EpCAM was demonstrated by FACS analysis of EpCAM expressing SW1398 colon cancer cells incubated with transfected COS-7 supernatants (figure 4A), whereas sCE2 did not bind the EpCAM expressing cells. Thus, the C28 moiety of C28-sCE2 mediated EpCAM binding. Furthermore, SW1398 cells were plated and incubated with the transfected COS-7 supernatants for 24 h. Hereafter, cells were stained with anti-myc antibody to detect bound fusion protein. C28-sCE2 was detected at the membrane of SW1398 cells, shown in figure 4B, whereas cells incubated with sCE2 were not stained.

***4A***




***4B***

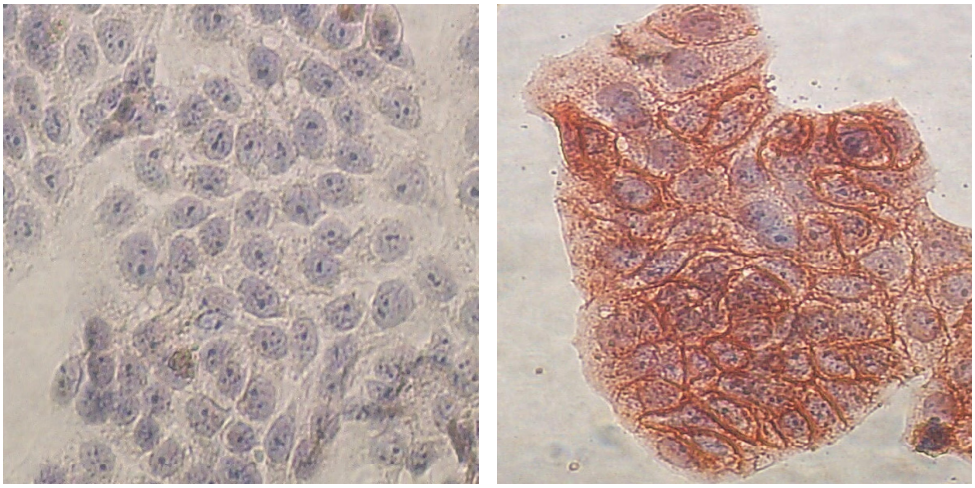


***Figure 4:*** *Binding of C28-sCE2 to the EpCAM expressing human colon cancer cell line SW1398. (A) FACS analysis of SW1398 cells that highly express EpCAM, with the supernatants of COS-7 cells transfected with psCE2 or pC28-sCE2. As a positive control the 323A3 antibody (bold line), directed to EpCAM was used. Binding was visualized with mouse anti-myc antibody and fluorescein-conjugated rabbit anti-mouse IgG. The fusion protein C28-sCE2 (dotted line) is able to bind to SW1398 cells, whereas sCE2 is overlapping the PBS control (solid line). (B) Cells were incubated for 24 h with supernatants of transfected COS-7 cells. Hereafter, cells were stained with anti-myc antibody to show binding of sCE2 or C28-sCE2 to EpCAM. Cells were counterstained with haematoxylin. Only cells incubated with C28-sCE2 (right) show binding of the fusion protein to the cellular membrane, whereas sCE2 incubation did not show bound protein (left).*

### *Prodrug activation and antiproliferative effects*

Concentrated supernatants of COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2 were analyzed for CPT-11 conversion using HPLC. Supernatants were incubated with CPT-11 for 22 h at 37°C. It was found that both sCE2 and C28-sCE2, which were secreted in the culture medium of transfected cells, were able to activate the prodrug CPT-11, since the drug SN-38 was formed (data not shown).

To show the effect of CPT-11 conversion into SN-38 by CE2, sCE2 and C28-sCE2 on the viability of colon cancer cells, the EpCAM-expressing colon carcinoma cell line SW1398 was incubated overnight with the concentrated supernatant of COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2. After incubation, culture medium or a non-toxic concentration (1 μM) of CPT-11 was added. In figure 5 it is shown that the supernatants of COS-7 cells transfected with sCE2 or C28-sCE2 render SW1398 cells susceptible to CPT-11. Incubation with supernatants of pCE2 transfected COS-7 cells and CPT-11, which do not secrete CE, or incubation with sCE2 or C28-sCE2 supernatant only, did not show augmented toxicity.

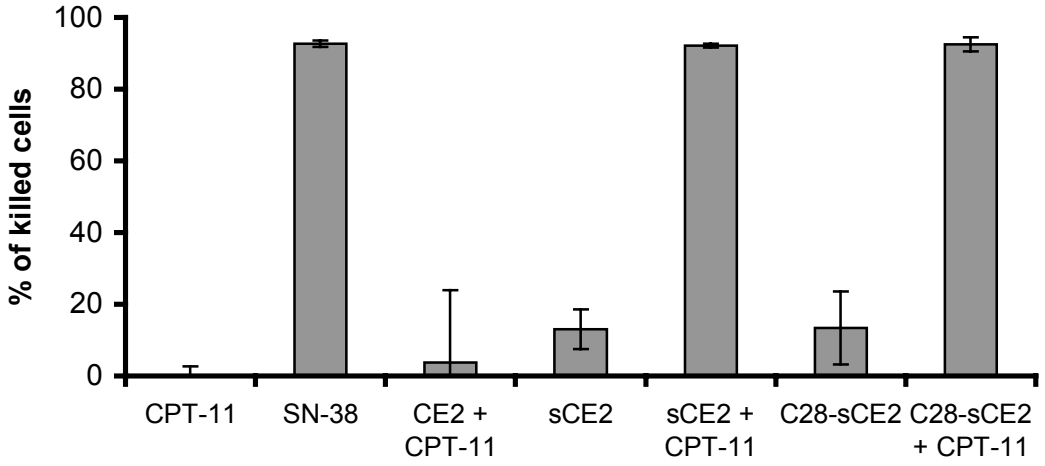



***Figure 5:*** *Cytotoxicity assay with the EpCAM expressing cell line SW1398 incubated with 1 μM CPT-11 and concentrated supernatants of COS-7 cells transfected with pCE2, psCE2 or pC28-sCE2 or with supernatants only. Results are shown as % of killed cells compared to untreated control cells, which were set to 0 % kill. Incubation with sCE2 or C28-sCE2 supernatants and the non-toxic concentration of CPT-11 result in growth inhibition comparable to incubation with 1 μM SN-38, whereas incubation with CPT-11 or supernatant only is not toxic.*

## DISCUSSION

CPT-11 is a prodrug for the treatment of colon cancer. One enzyme that converts CPT-11 into the toxic drug SN-38 is CE. By increasing the concentration of CE at the site of a tumor via a GDEPT approach, the conversion of CPT-11 to SN-38 will be enhanced at the site of the tumor, leading to tumor specific cytotoxicity. Human liver CE1 and rabbit CE have been employed in a GDEPT approach in combination with CPT-11. Although rabbit CE appeared to convert CPT-11 very effectively [17], an enzyme of human origin is preferred for *in vivo* applications to treat patients. Human CE1 showed a low conversion velocity and a low hydrolysis rate for CPT-11 in comparison with CE2 [19]. Therefore, in this study we used the human liver CE2 enzyme to sensitize human tumor cells to CPT-11. Because current gene transfer technology does not allow expression of transgenes in all cells of a targeted tumor *in vivo*, a bystander effect is required, in order to achieve

efficient tumor reduction. Extracellularly produced SN-38 should not only kill the tumor cells in which CE2 is formed, but also neighboring tumor cells that do not express *CE2*. To investigate whether extracellular conversion of CPT-11 would lead to a larger bystander effect than intracellular conversion, we constructed a secreted form of CE2 (sCE2). Furthermore, we hypothesized that a fusion protein consisting of sCE2 fused to a tumor specific scFv antibody would be retained in the tumor thereby preventing leakage of the enzyme into the circulation and therefore further reducing unwanted side effects. An example of a tumor-associated antigen is EpCAM. This molecule is an attractive target for enzyme prodrug therapy, since it is highly expressed on the cell surface of most carcinomas, including colon tumors. Furthermore, *EpCAM* is highly expressed on distant metastasis [28]. Therefore, we constructed a fully human fusion protein consisting of sCE2 fused to a human scFv antibody directed to EpCAM (C28-sCE2). Intratumoral expression of this protein in cancer patients is expected to be less immunogenic than expression of non-human fusion proteins.

The secreted and the targeted protein were detected in the supernatant of transfected COS-7 cells and the secreted proteins exhibited comparable enzymatic activities as determined by conversion of pNpAc. Comparing the secreted proteins to intracellular wild type CE2, it was observed that transfecting COS-7 cells with pCE2 resulted in a much lower total amount of CE-activity than cells transfected with psCE2 or the fusion protein C28-sCE2. Whether this is due to a greater amount of protein or a higher enzyme activity of sCE2 when compared with CE is not clear. C28-sCE2 showed enzyme activity and specific binding to EpCAM expressing cells as determined by FACS analysis and immunohistochemistry on SW1398 cells, whereas sCE2 did not bind these cells. Furthermore, using HPLC analysis it was shown that the secreted as well as the targeted form of CE2 were able to efficiently convert CPT-11 into SN-38. Experiments with SW1398 colon carcinoma cells that were incubated with secreted or targeted protein and a non-toxic concentration of CPT-11 showed complete growth inhibition of these cells.

In conclusion, we constructed a secreted form of CE2 that was capable to convert the prodrug CPT-11, leading to enhanced toxicity of CPT-11 to colon cancer cells. This construct holds promise in GDEPT approaches since transduction of tumor cells with psCE2 will most likely result in high concentrations of sCE2 throughout the whole tumor. Therefore, CPT-11 will be converted to SN-38 very efficiently throughout the tumor, resulting in a larger bystander effect than intracellular conversion of CPT-11. The C28-sCE2 fusion protein is as active as sCE2, and therefore this construct is as useful as sCE2 for GDEPT, but the theoretical advantage of C28-sCE2 is that the targeting moiety will prevent leakage of the construct into the circulation. However, from this study it can not be concluded that C28-sCE2 will have this additional advantage as compared to sCE2 .To prove this hypothesis, *in vivo* experiments are necessary in which *sCE2* and *C28-sCE2* are expressed in colon carcinoma xenografts followed by CPT-11 administration.

## ACKNOWLEDGEMENTS

The authors would like to thank Dr. Binfang Yan for kindly providing the CE2 antibody.

## REFERENCES

1 Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res* 1986; **46:** 5276-5281.

2 Austin EA, Huber BE. A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of Escherichia coli cytosine deaminase. *Mol Pharmacol* 1993; **43:** 380-387.

3 Satoh T *et al.* Metabolic activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1- piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. *Biol Pharm Bull* 1994; **17:** 662-664.

4 Tsuji T *et al.* CPT-11 converting enzyme from rat serum: purification and some properties. *J Pharmacobiodyn* 1991; **14:** 341-349.

5 Thompson J *et al.* Efficacy of oral irinotecan against neuroblastoma xenografts. *Anticancer Drugs* 1997; **8:** 313-322.

6 Thompson J *et al.* Efficacy of systemic administration of irinotecan against neuroblastoma xenografts. *Clin Cancer Res* 1997; **3:** 423-431.

7 Houghton PJ *et al.* Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemother Pharmacol* 1995; **36:** 393-403.

8 Houghton JA *et al.* Evaluation of irinotecan in combination with 5-fluorouracil or etoposide in xenograft models of colon adenocarcinoma and rhabdomyosarcoma. *Clin Cancer Res* 1996; **2:** 107-118.

9 Gupta E *et al.* Pharmacokinetic and pharmacodynamic evaluation of the topoisomerase inhibitor irinotecan in cancer patients. *J Clin Oncol* 1997; **15:** 1502-1510.

10 Rivory LP *et al.* Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. *Clin Cancer Res* 1997; **3:** 1261-1266.

11 Satoh T, Hosokawa M. The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* 1998; **38:** 257-288.

12 Ketterman AJ, Bowles MR, Pond SM. Purification and characterization of two human liver carboxylesterases. *Int J Biochem* 1989; **21:** 1303-1312.

13 Hojring N, Svensmark O. Carboxylesterases of human brain extract. Purification and properties of a butyrylesterase. *Biochim Biophys Acta* 1977; **481:** 500-514.

14 Munger JS *et al.* A serine esterase released by human alveolar macrophages is closely related to liver microsomal carboxylesterases. *J Biol Chem* 1991; **266:** 18832-18838.

15 Kojima A, Hackett NR, Ohwada A, Crystal RG. In vivo human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors. *J Clin Invest* 1998; **101:** 1789-1796.
16 Kojima A, Hackett NR, Crystal RG. Reversal of CPT-11 resistance of lung cancer cells by adenovirus-mediated gene transfer of the human carboxylesterase cDNA. *Cancer Res* 1998; **58:** 4368-4374.
17 Danks MK *et al.* Comparison of activation of CPT-11 by rabbit and human carboxylesterases for use in enzyme/prodrug therapy. *Clin Cancer Res* 1999; **5:** 917-924.
18 Wierdl M *et al.* Sensitization of Human Tumor Cells to CPT-11 via Adenoviral-mediated Delivery of a Rabbit Liver Carboxylesterase. *Cancer Res* 2001; **61:** 5078-5082.
19 Humerickhouse R *et al.* Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res* 2000; **60:** 1189-1192.
20 Sone T, Wang CY. Microsomal amidases and carboxylesterases. *Comprehensive Toxicology* 1997; **3:** 265-281.
21 Arafat W *et al.* Antineoplastic effect of anti-erbB-2 intrabody is not correlated with scFv affinity for its target. *Cancer Gene Ther* 2000; **7:** 1250-1256.
22 Huls GA *et al.* A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. *Nat Biotechnol* 1999; **17:** 276-281.
23 De Graaf M *et al.* A fully human anti-Ep-CAM scFv beta-glucuronidase fusion protein for selective chemotherapy with a glucuronide prodrug. *Brit J Cancer* 2002; **86:** 811-818.
24 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227:** 680-685.
25 Chan S *et al.* A novel tumour marker related to the c-myc oncogene product. *Mol Cell Probes* 1987; **1:** 73-82.
26 Zhu W *et al.* Dexamethasone differentially regulates expression of carboxylesterase genes in humans and rats. *Drug Metab Dispos* 2000; **28:** 186-191.
27 Edwards DP *et al.* Monoclonal antibody identification and characterization of a Mr 43,000 membrane glycoprotein associated with human breast cancer. *Cancer Res* 1986; **46:** 1306-1317.
28 Litvinov SV *et al.* Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J Cell Biol* 1994; **125:** 437-446.

# Chapter 3

# Adenoviral vector mediated expression of a gene encoding secreted, EpCAM targeted carboxylesterase-2 sensitizes colon cancer spheroids to CPT-11

Dinja Oosterhoff[1], Renée M. Overmeer[1], Michelle de Graaf[1], Ida H. van der Meulen[1], Giuseppe Giaccone[1], Victor W. van Beusechem[1], Hidde J. Haisma[2], Herbert M. Pinedo[1] and Winald R. Gerritsen[1]

[1] Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands
[2] Department of Therapeutic Gene Modulation, University Center for Pharmacy, P.O. Box 196, 9700 AD Groningen, The Netherlands

British Journal of Cancer 2005, 92: 882-887

## SUMMARY

CPT-11 is an anticancer agent in use for the treatment of colon cancer. In order to be fully active, CPT-11 needs to be converted into SN-38 by the enzyme carboxylesterase. In humans, only a minority of CPT-11 is converted to SN-38. To increase the anti-tumor effect of CPT-11 by Gene-Directed Enzyme Prodrug Therapy, we constructed a replication deficient adenoviral vector Ad.C28-sCE2 containing a fusion gene encoding a secreted form of human liver carboxylesterase-2 targeted to the surface antigen EpCAM that is highly expressed on most colon carcinoma cells. By targeting carboxylesterase-2 to EpCAM, the enzyme should accumulate specifically in tumors and leakage into the circulation should be minimized. Ad.C28-sCE2 transduced colon carcinoma cells expressed and secreted active carboxylesterase that bound specifically to EpCAM expressing cells. In sections of 3-dimensional colon carcinoma spheroids transduced with Ad.C28-sCE2, it was shown that C28-sCE2 was capable of binding untransduced cells. Most importantly, treatment of these spheroids with non-toxic concentrations of CPT-11 resulted in growth inhibition comparable to treatment with SN-38. Therefore, Ad.C28-sCE2 holds promise in gene therapy approaches for the treatment of colon carcinoma.

## INTRODUCTION

Conventional chemotherapy is not specific for tumor cells and therefore its administration is limited by side effects. These side effects might potentially be overcome by targeting chemotherapy specifically to tumor cells by Gene-Directed Enzyme Prodrug Therapy (GDEPT). In GDEPT, a gene encoding a prodrug-converting enzyme is delivered to the tumor by, for example, an adenoviral vector. If the prodrug is administered it will be specifically converted to the active drug at the site of the tumor. This should increase the efficacy and decrease the side effects of chemotherapy. CPT-11 (irinotecan or 7-ethyl-10[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) is an anticancer agent that is approved for first line treatment of metastatic colon cancer. In order to be fully active, CPT-11 needs to be activated into the active compound SN-38 (7-ethyl-10-hydroxycamptothecin) by carboxylesterase (CE) enzymes [1,2]. Although SN-38 is detected in the plasma of cancer patients only minutes after administration of CPT-11 [3], 90-95% of the prodrug is not converted to SN-38 [4]. A way to improve the anti-tumor effect of CPT-11 may be to use CPT-11 and CE in a GDEPT approach. Adenoviral mediated expression of rabbit *CE* showed to efficiently sensitize a panel of tumor cell lines to CPT-11 [5]. A human enzyme, however, has the advantage over a non-human enzyme that it will not lead to an immune response against the enzyme and subsequent enzyme inactivation. Kojima *et al*. described the construction of a replication deficient adenoviral vector containing the cDNA encoding human liver CE isoform 1 (CE1) [6]. Cell lines transduced with this virus and treated with CPT-11, however, showed only minimal anti-tumor effects. The liver CE isoform 2 (CE2) has a higher affinity and a higher conversion velocity of CPT-11 compared to CE1 [7]. Therefore, we envisaged that human liver CE2 would be the best candidate to employ in a

GDEPT approach to treat human colon cancer. Given the fact that current gene transfer technologies do not allow transduction of all tumor cells, a bystander effect is warranted to achieve effective kill of untransduced tumor cells. To improve the bystander effect of adenoviral vector mediated GDEPT approaches, secreted and surface-tethered prodrug converting enzymes have been investigated [8,9,10,11]. We envisioned that a targeted, secreted form of CE2, consisting of the secreted form of CE2 (sCE2) fused to a tumor specific scFv antibody would provide an enlarged bystander effect and would furthermore theoretically prevent leakage of the protein into the circulation, thereby reducing systemic side effects. Previously, we constructed a fusion protein in which sCE2 was fused to the human scFv antibody C28 which is directed to the tumor antigen Epithelial Cell Adhesion Molecule (EpCAM) [12]. This fusion protein has potential utility for GDEPT of colon cancer, because *EpCAM* is highly overexpressed in colon cancer cells including distant metastases [13]. Here, we describe the construction of a replication deficient adenoviral vector containing the cDNA encoding the fully human fusion protein C28-sCE2. In a 3-D tumor spheroid model *in vitro*, we could demonstrate that the secreted fusion protein bound non-transduced cells and caused efficient killing of colon cancer cells in the presence of CPT-11.

## MATERIALS AND METHODS

### *Cells and culture conditions*

The colon cancer cell lines SW1398 and Colo205 and the ovarian cancer cell line A2780 (all cell lines were kindly provided by Dr. E. Boven, VUMC, Amsterdam, The Netherlands) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 50 IU/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Breda, The Netherlands), at 37°C in a 5% $CO_2$ humidified atmosphere. The 293-cell line (ATCC, Manassas, VA) was maintained in DMEM supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2mM L-glutamine (Invitrogen).

### *Formation of colon cancer spheroids*

96-well plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 2% agarose (Roche, Almere, The Netherlands) in PBS. Colon cancer SW1398 or Colo205 cells were plated ($1.10^4$ cells/well) and rotated overnight at 140 rpm in a Heidolph Unimax incubator. By plating equal amounts of cells in each well and rotating them overnight, spheroids of similar sizes are formed. This allows direct comparison of different treatment modalities. After rotation, the formed spheroids were grown in a 5% $CO_2$ humidified atmosphere at 37°C for 3 days before use in transduction experiments.

### *Construction of Ad.C28-sCE2*

The adenoviral vector Ad.C28-sCE2 was constructed using the AdEasy System [14]. The plasmid pSTCF-C28-sCE2, containing the secreted, EpCAM-targeted CE2 (C28-sCE2) open reading frame [12] with a myc-6His tag at the C-terminus, was digested with *PmeI* and *NheI* and the *C28-sCE2* open reading

frame was ligated into the *XbaI* and *EcoRV* linearized transfer vector pAdTrack-CMV. This construct contains a gene encoding Green Fluorescent Protein (*GFP*) under the CMV promoter. Subsequently, the plasmid was digested with *PmeI* and cotransformed into *E. coli BJ5183* cells with adenoviral backbone plasmid pAdEasy-1 to construct pAdEasy-C28-sCE2. After linearization of this recombinant vector with *PacI*, the plasmid was transfected into the 293 adenovirus packaging cell line. Virus was further propagated in 293 cells according to standard techniques. For all experiments AdGFP [15] was taken along as a negative control.

***Western blot analysis***

Equivalent amounts of supernatant or cellular lysate from SW1398 cells transduced with Ad.C28-sCE2 were dissolved in sample buffer [16] with 2-mercaptoethanol and heated to 95°C for 5 min. Samples were electrophorezed through a denaturing 10% sodium dodecyl sulphate-polyacrylamide gel and protein bands were electroblotted onto a PVDF protein membrane (BioRad, Veenendaal, The Netherlands). Proteins were detected using anti-myc antibody 9E10 [17] and HRP-conjugated rabbit anti-mouse IgG (DakoCytomation, Heverlee, Belgium). Films were developed with enhanced chemoluminescence (Lumilight Plus, Roche).

***Esterase activity assay***

To evaluate the esterase activity of proteins expressed by SW1398 cells transduced with Ad.C28-sCE2, cellular lysates or supernatants were incubated with 200 μl 100 mM Tris-HCl pH =8.0 containing 1 mM p-nitrophenyl-acetate (pNpAc) (Sigma Aldrich, Zwijndrecht, The Netherlands), a substrate for CE. Conversion to pNp at room temperature was measured during 10 minutes using an ELISA plate reader (BioRad) at a wavelength of 415 nm.

***Immunohistochemistry***

Spheroids were harvested at different time points after transduction (day 1, 4 or 5) in TissueTek (Sakura Finetek, Zoeterwoude, The Netherlands) and cryostat sections of 7-10 μM were made and stored at -80°C. After drying, sections were fixed with 4% formaldehyde in PBS for 30 minutes, washed with PBS and treated with 0.2% Triton-X-100 in PBS. After washing, the sections were incubated for 1h with the anti-myc antibody 9E10. As a positive control, anti-EpCAM antibody 323A3 (kindly provided by Centocor, Leiden, the Netherlands) was taken along and as negative controls PBS/0.1% BSA and anti-glucuronidase [18] were used. After incubation, sections were washed with PBS and incubated with rabbit-anti-mouse-HRP or goat-anti-rabbit-HRP (1:100 in PBS/0.1% BSA, both from DakoCytomation). After incubation for 1 h, sections were washed with PBS and stained with AEC (DakoCytomation) and sections were counterstained with haematoxylin.

***In vitro cytotoxicity assays***

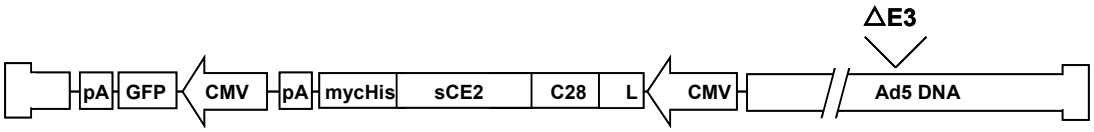
Three days after formation of colon cancer spheroids, the spheroids were transduced with $1.10^7$ plaque-forming units Ad.C28-sCE2 in 100 μl culture medium. Control spheroids were transduced with AdGFP or cultured in medium. After 7 days, 100 μl culture medium was added containing a range of CPT-11 (Aventis, Strasbourg, France). After a further 7 days, cell viability was determined by WST-1 (Roche Diagnostics) conversion at 37°C. Data are expressed as percentages compared to untransduced, untreated control spheroids.

## RESULTS

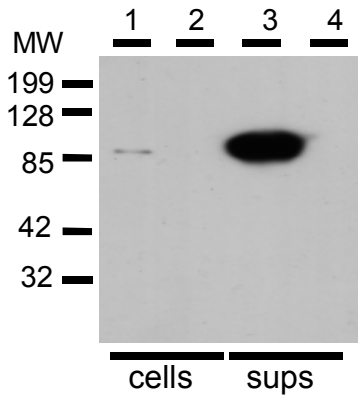
***Construction and characterization of Ad.C28-sCE2***

The open reading frame of EpCAM-targeted carboxylesterase C28-sCE2 with C-terminal mycHis-tag was inserted in place of the *E1* region of an adenovirus vector next to a GFP expression cassette to create Ad.C28-sCE2 (Figure 1A). SW1398 colon cancer cells were transduced with Ad.C28-sCE2 or control virus AdGFP at MOI 100 and after 6 days expression of *C28-sCE2* in supernatant and cellular lysate was analyzed by Western blotting. Figure 1B shows that the majority of the 110 kDa C28-sCE2 protein was detected in the supernatant of Ad.C28-sCE2 transduced cells, confirming efficient secretion. Enzyme activity of C28-sCE2 was demonstrated by an esterase enzyme activity assay (figure 1C). Binding of C28-sCE2 to EpCAM expressing cells was shown by immunohistochemistry (figure 1D). The EpCAM positive cell line Colo205 and the EpCAM negative ovarian cancer cell line A2780 were incubated with the supernatant of SW1398 cells transduced with Ad.C28-sCE2 or AdGFP. As can be seen in figure 1D, C28-sCE2 specifically bound to the cellular membranes of *EpCAM* expressing cells

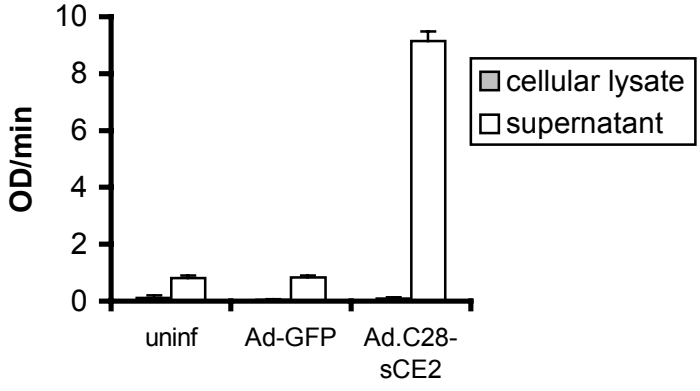
***1A***




***1B***




***1C***



***Diffusion of C28-sCE2 in multicellular colon cancer tumor spheroids***

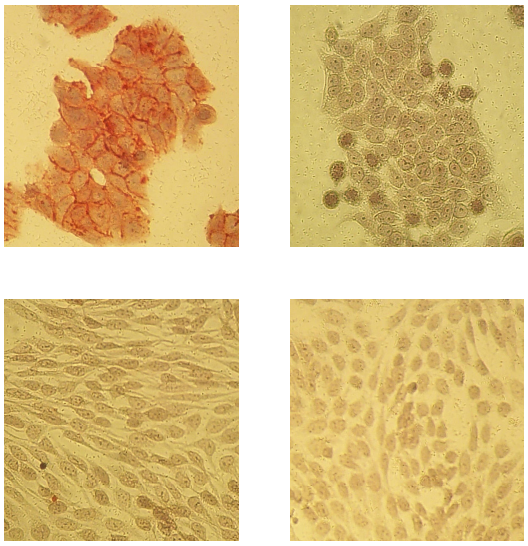
Colo205 spheroids were transduced with Ad.C28-sCE2 and cryosections were made 1, 4 and 5 days later. Sections were stained with an anti-myc antibody to localize the C28-sCE2 fusion protein. Figure 2 illustrates that on day 1 after transduction only the outer rim of the spheroid stained slightly positive for C28-sCE2. Sections of spheroids harvested at later time points after transduction showed presence of C28-sCE2 in deeper layers of the spheroid. A higher magnification of the anti-myc staining at day 5 after transduction (figure 2B) suggests that C28-sCE2 had bound untransduced neighboring cells since only the cellular membrane of these cells stained positive. Thus, C28-sCE2 penetrated into and accumulated in the tumor mass surrounding Ad.C28-sCE2 transduced cells.

***CPT-11 activation and antiproliferative effects in Ad.C28-sCE2 transduced cells***

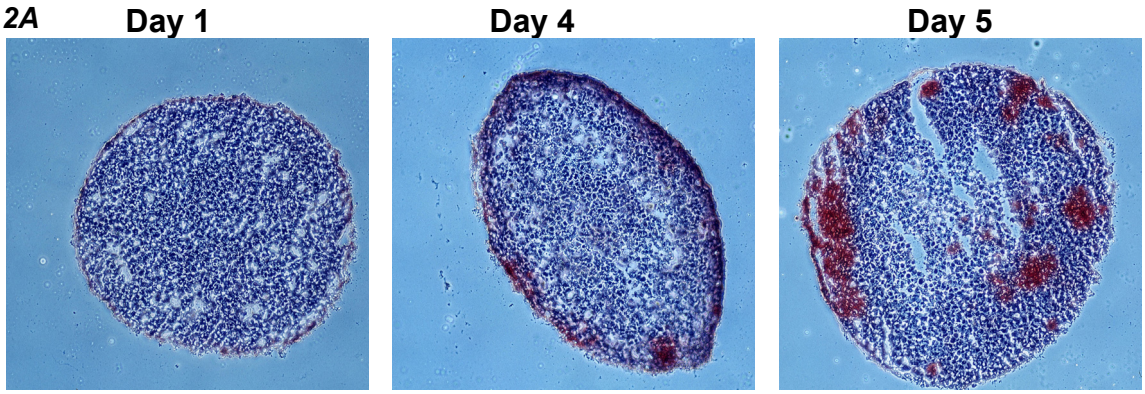
Colon cancer spheroids transduced with Ad.C28-sCE2 or AdGFP were subjected to CPT-11 treatment for 7 days. Figure 3 demonstrates the viability of the spheroid as measured by WST-1 conversion. Ad.C28-sCE2 transduced Colo205 and SW1398 colon cancer spheroids were sensitized to CPT-11, since CPT-11 treatment to these spheroids was as toxic as treatment with its activated analogue SN-38.

***Figure 1:*** *Schematic structure of the replication deficient adenovirus Ad.C28-sCE2 and characterization of Ad.C28-sCE2 transduced SW1398 cells by Western blot analysis, esterase activity assay and immunohistochemistry. (A) Schematic structure of the replication deficient adenovirus Ad.C28-sCE2. The C28-sCE2 expression cassette includes the CMV promoter, an IgG$\kappa$ leader sequence for secretion and a C-terminal myc- and His-tag for detection and purification. The adenovirus also contains the gene encoding GFP under the CMV promoter. (B) Western blot analysis of cellular lysates (lane 1 and 2) and supernatants (lane 3 and 4) of SW1398 cells transduced with Ad.C28-sCE2 (lane 1 and 3) or AdGFP (lane 2 and 4) at MOI 100. C28-sCE2 was detected using an antibody directed to the myc-tag. (C) CE-activity in cellular lysates and supernatants of SW1398 cells transduced with Ad.C28-sCE2 or AdGFP at MOI 100. Cellular lysates or supernatants were incubated with 1mM pNpAc and conversion was measured during 10 minutes. C28-sCE2 showed enzymatic activity and was efficiently secreted by transduced cells, since most of the activity was detected in the supernatant. (D) Binding of C28-sCE2 to the EpCAM expressing cell line Colo205. Colo205 cells or the EpCAM negative cell line A2780 were incubated with the supernatant of SW1398 cells transduced with Ad.C28-sCE2 or AdGFP at MOI 100. After washing, the cells were stained with anti-myc antibody to show binding of C28-sCE2. Only the EpCAM expressing Colo205 cells incubated with supernatant of Ad.C28-sCE2 transduced SW1398 cells showed a positive membrane staining, indicating that the fusion protein had bound specifically to the Colo205 cells. Figure 1D is shown at page 82.*

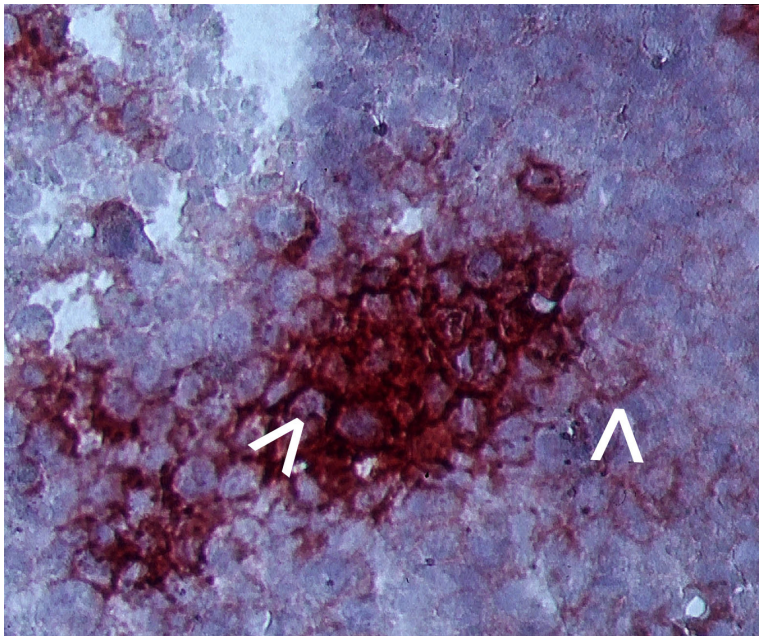
***1D***




***2A***




***2B***



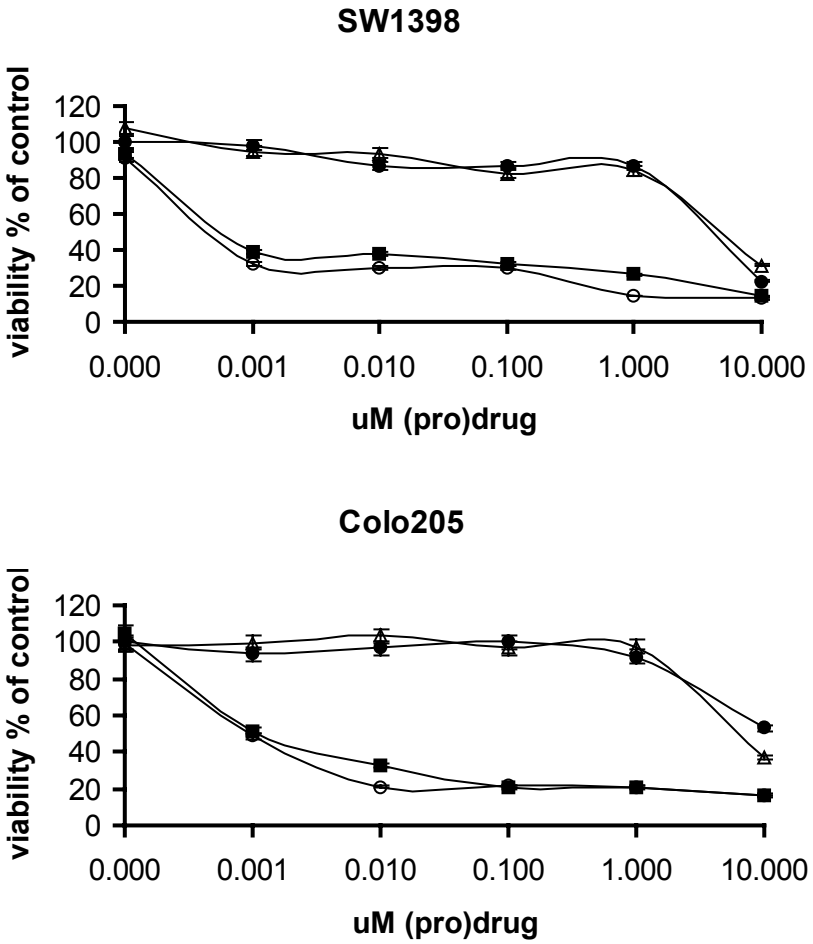



***Figure 3:*** *Cytotoxicity assay on SW1398 and Colo205 colon cancer spheroids. SW1398 (A) and Colo205 (B) spheroids were transduced with 1.10$^7$ pfu AdGFP or Ad.C28-sCE2. Seven days after infection spheroids were subjected to a range of CPT-11 concentrations and cultured for a further 7 days. Cell viability of untransduced spheroids treated with CPT-11 (closed black circles) or SN-38 (open black circles), AdGFP transduced spheroids treated with CPT-11 (open black triangles) and Ad.C28-sCE2 transduced spheroids treated with CPT-11 (closed black squares) were analyzed by WST-1 conversion measurement.*

***Figure 2:*** *Immunohistochemistry on sections of Ad.C28-sCE2 transduced Colo205 spheroids. Colo205 spheroids were transduced with 1.10$^7$ pfu Ad.C28-sCE2 and harvested at day 1, day 4 and day 5 after transduction. Sections of these spheroids were made and stained for myc to detect C28-sCE2. (A) At day 1 after Ad.C28-sCE2 transduction no positive staining can be detected. At days 4 and 5, several spots along the rim of the spheroid are positively stained. (B) A higher magnification of the fusion protein staining at day 5 after transduction is shown. Cells with clear staining of membranes only (arrows) represent untransduced neighboring cells with bound C28-sCE2.*

## DISCUSSION

Targeting chemotherapy specifically to tumor cells with GDEPT is expected to increase the anti-tumor effect, while side effects are decreased. A limitation of adenoviral vector mediated cancer gene therapy is the poor penetration ability of adenoviral vectors into a solid tumor mass. To improve the efficacy of adenoviral vector mediated GDEPT approaches, secreted prodrug converting enzymes have been studied [8,9]. However, secreted enzymes might leak away from the site of the tumor. Therefore, cell surface-tethered forms of prodrug converting enzymes, such as β-glucuronidase or carboxypeptidase G2, were developed to prevent leakage of untargeted enzyme from the tumor, while prodrug activation is retained [10,11]. Another way to prevent diffusion of the enzyme from the tumor is secretion by transduced tumor cells of a fusion protein consisting of a scFv antibody and a prodrug-converting enzyme, which can subsequently bind to tumor cells [12,19]. We hypothesized that the bystander effect achieved by such a secreted targeted prodrug converting enzyme might be more pronounced than that achieved by a cell surface-tethered form, as the targeted form can diffuse and bind to neighboring tumor cells. In this study, we investigated the utility of a replication deficient adenoviral vector containing the cDNA encoding a secreted, EpCAM-targeted form of human liver carboxylesterase-2, Ad.C28-sCE2, to sensitize colon cancer tumors to CPT-11. We chose to study Ad.C28-sCE2 in a 3-dimensional *in vitro* colon cancer spheroid model, because the three-dimensional structure of spheroids resembles *in vivo* tumors much closer than two-dimensional cell cultures. Furthermore, we wanted to visualize the bystander effect by determining secretion of C28-sCE2 and penetration of the fusion protein through a solid tumor mass, which can only be studied in a 3-dimensional structure. Grill *et al*. demonstrated that transduction of primary glioma spheroids with a replication deficient vector resulted in expression of the transgene in the outer rim of the spheroid only. This showed that spheroids are relevant structures to study lack of adenovirus penetration into solid tumor masses [20]. In the colon cancer spheroid model used in this study, we were able to detect the C28-sCE2 fusion protein bound to untransduced cells several cellular layers away from transduced cells. This suggests that C28-sCE2 is capable of diffusing into a solid tumor mass.

From these results we hypothesized that optimal cytotoxicity from CPT-11 could be expected if the prodrug was administered at least a few days after Ad.C28-sCE2 transduction when C28-sCE2 has spread through the spheroid. Transduction of colon cancer spheroids with Ad.C28-sCE2 and treatment with CPT-11 after 7 days resulted in complete sensitization of these spheroids to CPT-11. The toxicity to these spheroids was comparable to SN-38 treatment, indicating that CPT-11 is effectively converted into the toxic drug.

In order to compare a targeted prodrug-converting enzyme with a secreted prodrug-converting enzyme it is necessary to perform *in vivo* experiments. However, the high endogenous plasma esterase activity in mice presents a challenge in using mouse models to evaluate tumor-specific conversion of CPT-11. In mice, more than 50% of the administered CPT-11 is converted to SN-38 by plasma esterases [21], whereas in human patients less than 5% of the prodrug is activated [4]. Hence, the analysis of CE-mediated activation of CPT-11 in normal mice does not accurately reflect what happens after

administration of the drug to humans. Previously, a strain of plasma esterase-deficient mice was described [21], in which CPT-11 metabolism is comparable to that observed in humans. Recently, these mice were crossbred with SCID mice (personal communication with Dr. Phil Potter, St. Jude Children's Research Hospital, Memphis, USA) and we are currently testing adenoviral vectors expressing EpCAM-targeted sCE2 or untargeted sCE2 in these esterase deficient SCID mice bearing colon cancer xenografts.

In conclusion, we constructed a replication deficient adenoviral vector containing a cDNA encoding a secreted, EpCAM targeted form of human liver carboxylesterase-2 that was capable of converting the prodrug CPT-11 into its activated form, leading to enhanced toxicity of CPT-11 to colon cancer spheroids. Therefore, this adenoviral construct holds promise in GDEPT approaches for the treatment of patients with EpCAM expressing colon cancer.

## ACKNOWLEDGEMENTS

The authors thank Jeroen Mastenbroek for his expert technical assistance. Dinja Oosterhoff is supported by an Educational Grant of Aventis. Victor van Beusechem is supported by a research fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW).

## REFERENCES

1 Satoh T *et al.* Metabolic activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1- piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. *Biol Pharm Bull 1994*; **17:** 662-664.

2 Tsuji T *et al.* CPT-11 converting enzyme from rat serum: purification and some properties. *J Pharmacobiodyn 1991*; **14:** 341-349.

3 Gupta E *et al.* Pharmacokinetic and pharmacodynamic evaluation of the topoisomerase inhibitor irinotecan in cancer patients. *J Clin Oncol 1997*; **15:** 1502-1510.

4 Rivory LP *et al.* Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. *Clin Cancer Res 1997*; **3:** 1261-1266.

5 Wierdl M *et al.* Sensitization of Human Tumor Cells to CPT-11 via Adenoviral-mediated Delivery of a Rabbit Liver Carboxylesterase. *Cancer Res 2001*; **61:** 5078-5082.

6 Kojima A, Hackett NR, Ohwada A, Crystal RG. In vivo human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors. *J Clin Invest 1998*; **101:** 1789-1796.

7 Humerickhouse R *et al.* Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res 2000*; **60:** 1189-1192.

8 Oosterhoff D *et al.* Gene-directed enzyme prodrug therapy for osteosarcoma: sensitization to CPT-11 in vitro and in vivo by adenoviral delivery of a gene encoding secreted carboxylesterase-2. *Mol Cancer Ther 2003*; **2:** 765-771.

9 Weyel D, Sedlacek HH, Muller R, Brusselbach S. Secreted human beta-glucuronidase: a novel tool for gene-directed enzyme prodrug therapy. *Gene Ther 2000*; **7:** 224-231.
10 Spooner RA *et al.* In suicide gene therapy, the site of subcellular localization of the activating enzyme is more important than the rate at which it activates prodrug. *Cancer Gene Ther 2000*; **7:** 1348-1356.
11 Heine D, Muller R, Brusselbach S. Cell surface display of a lysosomal enzyme for extracellular gene- directed enzyme prodrug therapy. *Gene Ther 2001*; **8:** 1005-1010.
12 Oosterhoff D *et al.* Secreted and tumor targeted human carboxylesterase for activation of irinotecan. *Br J Cancer 2002*; **87:** 659-664.
13 Litvinov SV *et al.* Evidence for a role of the epithelial glycoprotein 40 (Ep-CAM) in epithelial cell-cell adhesion. *Cell Adhes Commun 1994*; **2:** 417-428.
14 He TC *et al.* A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A 1998*; **95:** 2509-2514.
15 van Beusechem VW *et al.* Recombinant adenovirus vectors with knobless fibers for targeted gene transfer. *Gene Ther 2000*; **7:** 1940-1946.
16 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 1970*; **227:** 680-685.
17 Chan S *et al.* A novel tumour marker related to the c-myc oncogene product. *Mol Cell Probes 1987*; **1:** 73-82.
18 Haisma HJ *et al.* A monoclonal antibody against human beta-glucuronidase for application in antibody-directed enzyme prodrug therapy. *Hybridoma 1995*; **14:** 377-382.
19 De Graaf M *et al.* A fully human anti-Ep-CAM scFv beta-glucuronidase fusion protein for selective chemotherapy with a glucuronide prodrug. *British Journal of Cancer 2002*; **86:** 811-819.
20 Grill J *et al.* The organotypic multicellular spheroid is a relevant three-dimensional model to study adenovirus replication and penetration in human tumors in vitro. *Mol Ther 2002*; **6:** 609-614.
21 Morton CL *et al.* Activation of CPT-11 in mice: identification and analysis of a highly effective plasma esterase. *Cancer Res 2000*; **60:** 4206-4210.

# Chapter 4

# Gene-Directed Enzyme Prodrug Therapy for Osteosarcoma: Sensitization to CPT-11 *in vitro* and *in vivo* by adenoviral delivery of a gene encoding secreted carboxylesterase-2

Dinja Oosterhoff[1], M. Adhiambo Witlox[2], Victor W. van Beusechem[1], Hidde J. Haisma[3], Gerard R. Schaap[4], Johannes Bras[5], Frank A. Kruyt[1], Bonnie Molenaar[1], Epie Boven[1], Paul I.J.M. Wuisman[2], Herbert M. Pinedo[1] and Winald R. Gerritsen[1]

Division of Gene Therapy, Departments of Medical Oncology[1] and Orthopaedics[2], VU University Medical Center, Amsterdam, The Netherlands
[3] Department of Therapeutic Gene Modulation, University Center for Pharmacy, Groningen, The Netherlands
Departments of Orthopedic Surgery[4] and Pathology[5], Academical Medical Center, Amsterdam, The Netherlands

Molecular Cancer Therapeutics 2003, 2: 765-771

## SUMMARY

Despite improvement in the treatment of osteosarcoma there are still many patients who cannot benefit from current treatment modalities. This warrants exploration of new treatment options. To that end, we investigated Gene-Directed Enzyme Prodrug Therapy (GDEPT) with the use of human liver carboxylesterase-2 (CE2) and the anticancer agent CPT-11. CPT-11 is a clinically approved prodrug that needs to be metabolized into the active drug SN-38 by carboxylesterases, which occurs rather inefficiently in humans. GDEPT aims at high production of CE2 at the tumor site resulting in efficient local conversion of CPT-11 into SN-38. We showed that osteosarcoma cells transduced with an adenoviral vector containing the cDNA encoding a secreted form of CE2 (Ad-sCE2) expressed and efficiently secreted *CE2*. *In vitro*, transduction of a panel of osteosarcoma cell lines with Ad-sCE2 resulted in sensitization up to 2800-fold to CPT-11 treatment. Primary osteosarcoma short-term cultures, derived from patients suffering from a classic high-grade osteosarcoma, demonstrated increased CPT-11 sensitivity up to 70-fold after transduction with Ad-sCE2 *in vitro*. When mice bearing subcutaneous MG-63 osteosarcoma xenografts were intratumorally injected with Ad-sCE2 and CPT-11, this resulted in a significant difference in time to reach 2000 mm3 in tumor volume as compared to animals receiving Ad-sCE2 or CPT-11 treatment ($p<0.05$).

Together, these data suggest that osteosarcoma cells are sensitive for the combination of Ad-sCE2 and CPT-11.


## INTRODUCTION

Osteosarcoma (OS) is the most common primary bone tumor in children and young adults [1]. Despite recent improvement in the treatment of OS there are still too many patients who cannot benefit from current treatment modalities [2]. The overall survival of primary OS with combined treatment, consisting of neoadjuvant chemotherapy and surgery, now varies between 50-65% [3-5]. Attempts to treat OS with multiple new agents have not increased survival rates. Therefore, new treatment options have to be explored for this type of disease.

Camptothecin derivatives have been explored in phase I-II trials including patients with OS refractory to standard treatment protocols. In these trials partial responses have been observed [6], [7]. Pratesi *et al.* have shown complete tumor response and cures in 70% of animals bearing subcutaneous U2OS OS. The tumor variant selected for resistance against cisplatin was still responsive to camptothecin treatment [8].

CPT-11 is a semi-synthetic, water-soluble derivative of camptothecin that differs from other camptothecin analogues, in that it is a prodrug that undergoes de-esterification to the much more potent topoisomerase I inhibitor, SN-38 (7-ethyl-10 hydroxycamptothecin) [9]. A class of enzymes that converts CPT-11 to SN-38 is the human carboxylesterases (CE). CE-activity can be detected in human liver, intestines and other sites [10]. Although patients do express *CE*, the amount of CPT-11 administered systemically that is converted to the active drug SN-38 is in the range of only 5 to 10% [11].

Furthermore, the usefulness of CPT-11 is hampered by dose-dependent toxicity, primarily diarrhea. A strategy to increase the antitumor effects of CPT-11, while decreasing the unwanted side effects, is to express the cDNA encoding CE specifically at the site of the tumor. After systemic administration of CPT-11, this will lead to tumor-specific conversion and therefore tumor-specific toxicity. This approach is called Gene-Directed Enzyme Prodrug Therapy (GDEPT). Another GDEPT approach previously described for OS in experimental models utilizes the viral enzyme thymidine kinase (TK) in combination with the prodrug acyclovir (ACV). The efficacy of this approach was shown *in vitro* [12] as well as *in vivo* [13] [14]. In the latter case, 80% of gene therapy treated animals bearing subcutaneous OS xenografts survived, whereas survival rates increased to 100% when gene therapy was combined with metrotrexate. The advantage of using CE/CPT-11 GDEPT approaches is that human isoforms of CE can be used which should not result in an immunological response and subsequent enzyme inactivation. Therefore, repeated administration should be possible.

We have screened a panel of human malignant cell lines originating of different tissues to explore which cell lines could be sensitized to CPT-11 by addition of CE into the culture medium. All osteosarcoma cell lines tested became highly sensitive for CPT-11 in the presence of extracellular CE (unpublished results). To explore the effect of CPT-11 on osteosarcoma cells in a GDEPT approach, we constructed an adenoviral vector containing the cDNA encoding secreted human carboxylesterase isoform-2 (Ad-sCE2). A secreted form of CE2 might have the advantage that it will likely spread through a solid tumor mass, resulting in extracellular conversion of CPT-11. This may lead to antitumor effects to untransduced neighboring tumor cells, also designated as the 'bystander effect'.

In this study, we describe the construction and characterization of the Ad-sCE2 adenoviral vector and its ability to sensitize osteosarcoma cell lines and primary osteosarcoma cells to CPT-11 *in vitro*. In addition, we report on the usefulness of Ad-sCE2 in human OS xenografts grown in nude mice and treated with CPT-11.

## MATERIALS AND METHODS

### *Cells and Culture conditions*

MG-63 [15] (courtesy of Dr. C. Löwik, Leiden University Medical Center, the Netherlands), MNNG-HOS [16], SaOs-2 [17], (courtesy of Dr. F. van Valen, Westfalische Wilhelms-Universität, Münster, Germany), CAL-72 [18] (courtesy of Dr. J. Gioanni, Laboratoire de Cancerologie, Faculté de Medicine, Nice, France) and HEK293 (ATCC, Manassas, VA) cell lines were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (all from Gibco BRL, Life Technologies B.V., Breda, The Netherlands), at 37°C in a 5% $CO_2$ humidified atmosphere.

***Patient material***

Fresh tumor material was obtained from patients having a classic high grade OS and was brought into culture as described [19]. From patient 6, cells were cultured before (OS-6) and after (OS-6a) chemotherapy. From patient 1, only material after chemotherapy was cultured (OS-1a). All experiments were performed in cell cultures at passage 0-5.

***Construction of Ad-sCE2***

The adenoviral vector Ad-sCE2 was constructed using the AdEasy method [20]. The plasmid pSTCF-sCE2 containing the secreted *CE2 (sCE2*) open reading frame with a myc-6xHis tag at the C-terminal ending [21], was digested with *PmeI* and *NheI* and the *sCE2* comprising fragment was cloned into *XbaI* and *EcoRV* digested pAdTrack-CMV. The resulting plasmid was digested with *PmeI* and recombined with adenoviral backbone plasmid pAdEasy-1 in *E. coli* BJ5183 cells to construct pAdEasy-sCE2. After linearization with *PacI*, the plasmid was transfected into the adenovirus packaging cell line HEK293 and virus was further propagated in HEK293 cells according to standard techniques. Purified virus stocks were prepared by two successive bandings on CsCl gradients. Viral particle (vp) titer and plaque forming unit (pfu) titer was determined by $OD_{260}$ and limiting dilution, respectively. The pfu titer was $1.5*10^{12}$ and the vp/pfu ratio was 18.1. For all experiments the pfu titer was used and Ad-GFP was taken along as a negative control [22].

***Western blot***

Equivalent amounts of supernatant or cellular lysate from SaOs-2 cells infected with Ad-sCE2 or Ad-GFP (multiplicity of infection (MOI) 100) were dissolved in sample buffer [23] with 5% 2-mercapto-ethanol and boiled at 95°C for 5 min. Samples were subjected to electrophoresis through a denaturing 10% sodium dodecyl sulphate-polyacrylamide gel and protein bands were electroblotted onto PVDF protein membrane (BioRad, Veenendaal, The Netherlands). Proteins were detected using mouse anti-myc antibody 9E10 [24] and horseradish peroxidase-conjugated rabbit anti-mouse IgG (DakoCytomation, Heverlee, Belgium). Blots were developed with enhanced chemoluminescence (Lumilight Plus, Roche Diagnostics, Almere, The Netherlands).

***Esterase activity***

Cellular lysates or supernatants of SaOs-2 cells infected with Ad-sCE2 or Ad-GFP were incubated with 200 μl 100 mM TRIS-HCl pH=8.0 containing 1 mM p-nitrophenyl-acetate (pNpAc) (Sigma Aldrich, Zwijndrecht, The Netherlands), a substrate for CE. Conversion to paranitrophenol was monitored over a 10-min period using an ELISA plate reader (BioRad, Veenendaal, The Netherlands) at a wavelength of 415 nm at room temperature.

***In vitro proliferation assay***

OS cell lines and primary OS cell cultures were plated in a 96-wells microtiter plate (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) at $5.10^3$ cells per well in 100 μl. After 24 h, medium was removed and cells were infected with Ad-sCE2 at an MOI of 1, 10 or 100 in 50 μl in culture medium with 2.5% FCS. After 1 h, virus was removed and 200 μl culture medium with or without CPT-11 (Aventis, Strasbourg, France) in a range of concentrations was added to separate wells in triplicate. Control experiments were performed in which the OS cells were infected with Ad-GFP and treated with CPT-11 or cells were only treated with CPT-11 or SN-38 (Aventis). After an incubation period of 6 days, growth was determined by incubating the cells with the cell proliferation reagent WST-1 (Roche Diagnostics) at 37°C. After 2 h the absorbency was measured at a wavelength of 450 nm using an ELISA plate reader. The antiproliferative effects of treated cells were expressed as percentages of growth from uninfected, untreated control cells.

***Crystal violet staining***

In a separate assay, cytotoxicity in OS cell lines and primary OS cell cultures was measured by crystal violet staining. Cells were treated as described for the antiproliferative assay. At the end of the experiment, medium was aspirated from cell cultures and adherent cells were fixed with 4% formaldehyde in PBS for a 10-minute period at room temperature. After fixation, cells were washed and incubated for 15 minutes with 1% crystal violet dissolved in 70% ethanol. Hereafter, cells were washed with water, air-dried and scanned on a BioRad GS690 scanner.

***In vivo experiments***

The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act (1977) and the published 'Guidelines on the protection of experimental animals' by the council of the E.C. (1986). The protocol was approved by the committee on Animal Research of the Vrije Universiteit.

Female athymic nu/nu mice, weighing 25-35 g, obtained from Harlan-CPB (Austerlitz, the Netherlands) were housed under pathogen-free conditions and were fed ad libitum. Tumor pieces (3x3x3 mm), derived from subcutaneously injected MG-63 cells in previous recipients, were implanted under the skin of one flank in anesthetized mice. Mice were weighed and tumor size was monitored twice a week using digital calipers. The tumor volume was calculated from the average of tumor length and width according to the formula: $4/3\Pi r^3$. The volume doubling time of untreated MG-63 tumors was 1.6 days.

In order to determine the highest dosage of CPT-11 that had no effect on tumor growth, mice were divided in 4 groups of 3 animals each, when the tumor nodules reached 200-300 $mm^3$. Mice in each group were intratumorally injected with 0.143 or 1.43 or 14.3 μg CPT-11 in 25 μl PBS or PBS alone for 7 consecutive days. Tumors were measured for a period of 3 weeks.

In the next experiment, mice were divided into three groups when the tumor nodules reached 200-300 $mm^3$ (designated as day 0). Tumors were injected with $1.10^9$ plaque forming units (pfu) of Ad-sCE2 in 25 μl PBS followed by

intratumoral injection with 1.43 μg CPT-11 in 25 μl PBS for 7 consecutive days (9 animals). Tumors of control mice received PBS on day 1 followed by injection with 1.43 μg CPT-11 for 7 consecutive days (6 mice) or $1.10^9$ pfu of Ad-sCE2 on day 1 followed by injection with PBS for 7 days (6 mice). The mice were euthanized when the tumors reached a size of ≥2000 $mm^3$. The different treatment modalities were evaluated by comparing tumor growth delay (time required to reach 5 times the initial tumor volume) and comparing the time for the tumors to reach a volume of ≥2000 $mm^3$, which was regarded as the endpoint of the Kaplan-Meier curves.

Statistical analyses were performed using SPSS (SPSS inc., Chicago, USA). The ANOVA test was used to compare the growth delay between the different treatment groups. The Kaplan-Meier curves were calculated and differences in time for the tumors to reach ≥2000 $mm^3$ between different treatment groups were analyzed using the log-rank test.

## RESULTS

### *Antiproliferative effects of CPT-11 and SN-38*

To assess the sensitivity of OS cells to CPT-11 and SN-38, IC50 values (the concentrations of prodrug or drug that results in 50% growth inhibition) were determined. OS cell lines and short-term cultures, derived from tumors of patients with classic high grade OS, were subjected to a range of concentrations of CPT-11 or SN-38 for 6 days. The IC50 values for CPT-11 and SN-38 and the ratios of these values are shown in Table 1. The OS cell lines tested were 625- to 1200-fold more sensitive to SN-38 than to CPT-11. In three primary OS cultures, this varied between 30- to 125-fold. For the other 3 short-term primary cultures tested the ratio was at least >33 times, but could not be determined exactly since the highest concentration of CPT-11 used was not toxic to these cells. These results indicate that efficient activation of CPT-11 to SN-38 would result in considerable antiproliferative effects in OS cells.

### *Construction and characterization of Ad-sCE2*

A secreted form of human liver CE2 (sCE2) was obtained by deletion of the cellular retention signal HTEL and by cloning it downstream of an IgGκ leader sequence. At the C-terminus a myc-6xHis tag was cloned for easy detection and purification. The expression of *sCE2* was driven by the cytomegalovirus (CMV) promoter [21]. The adenovirus vector Ad-sCE2, was constructed by inserting the *sCE2* expression cassette adjacent to a Green Fluorescent Protein (GFP) expression cassette in place of the adenovirus *E1* region (Figure 1).

| Name | Cell type | CPT-11 (IC50 in µM) | SN-38 (IC50 in µM) | Ratio* | Ad-GFP + CPT-11 (IC50 in µM) | Ad-sCE2 + CPT-11 (IC50 in µM) | d.o.s.** |
|---|---|---|---|---|---|---|---|
| SaOs-2 | Cell line | 2 | 0.0025 | 800 | 2 | 0.0007 | 2857 |
| MG-63 | Cell line | 3.5 | 0.003 | 1166 | 2.8 | 0.02 | 175 |
| CAL-72 | Cell line | 2.5 | 0.004 | 625 | 4 | 0.03 | 83.3 |
| MNNG-HOS | Cell line | 4 | 0.004 | 1000 | 4 | 0.4 | 10*** |
| OS-1A | Primary | 3 | 0.1 | 30 | 5 | 0.4 | 7.5 |
| OS-2 | Primary | >100 | 3 | >33.3 | >100 | 9 | >11.1 |
| OS-6 | Primary | >100 | 2 | >50 | >100 | 30 | >3.3 |
| OS-6A | Primary | >100 | 1.5 | >66.6 | >100 | 1.5 | >66.6 |
| OS-7 | Primary | 100 | 0.8 | 125 | 100 | 11 | 9 |
| OS-8 | Primary | 20 | 0.2 | 100 | 50 | 0.3 | 66.6 |

***Table 1:*** *Antiproliferative effects of CPT-11 and SN-38 and for CPT-11 after transduction with Ad-sCE2 or Ad-GFP in OS cells.*
** The ratio is expressed as IC50 value for CPT-11 divided by the IC50 value for SN-38.*
*** The degree of sensitization (d.o.s.) is expressed as IC50 value for CPT-11divided by the IC50 value for CPT-11 after cellular transduction with Ad-sCE2.*
**** The transduction efficiency of the MNNG-HOS cell line was < 5%, which could explain the moderate d.o.s.*

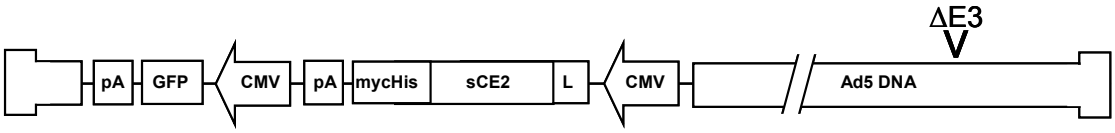



***Figure 1:*** *Schematic presentation of the adenovirus Ad-sCE2 that was constructed by inserting the sCE2 expression cassette adjacent to a GFP expression cassette in place of the adenovirus E1 region. The sCE2 expression cassette includes the CMV promoter (CMV), an IgG kappa leader sequence for secretion (L), a C-terminal myc- and 6xHis-tag for detection and purification (mycHis) and an SV40 polyadenylation signal (PA).*

SaOs-2 cells were infected with either Ad-sCE2 or control virus Ad-GFP (MOI 100) and after 6 days supernatant and cellular lysates were analyzed for *sCE2* expression by Western blot. Figure 2A shows that almost all sCE2 was detected in the supernatant of infected cells. Functional activity of sCE2 at day 6 after transduction was demonstrated by an esterase activity assay (Figure 2B). Again, most of the CE activity was detected in the supernatant of SaOs-2 cells infected with Ad-sCE2, confirming the results of the Western blot.

To follow sCE2 secretion in the supernatant of Ad-sCE2 transduced cells in time, SaOs-2 supernatants were harvested at day 1 to 6 after transduction and a CE activity assay was performed (figure 2C). A gradual increase of CE activity in time after transduction was observed.

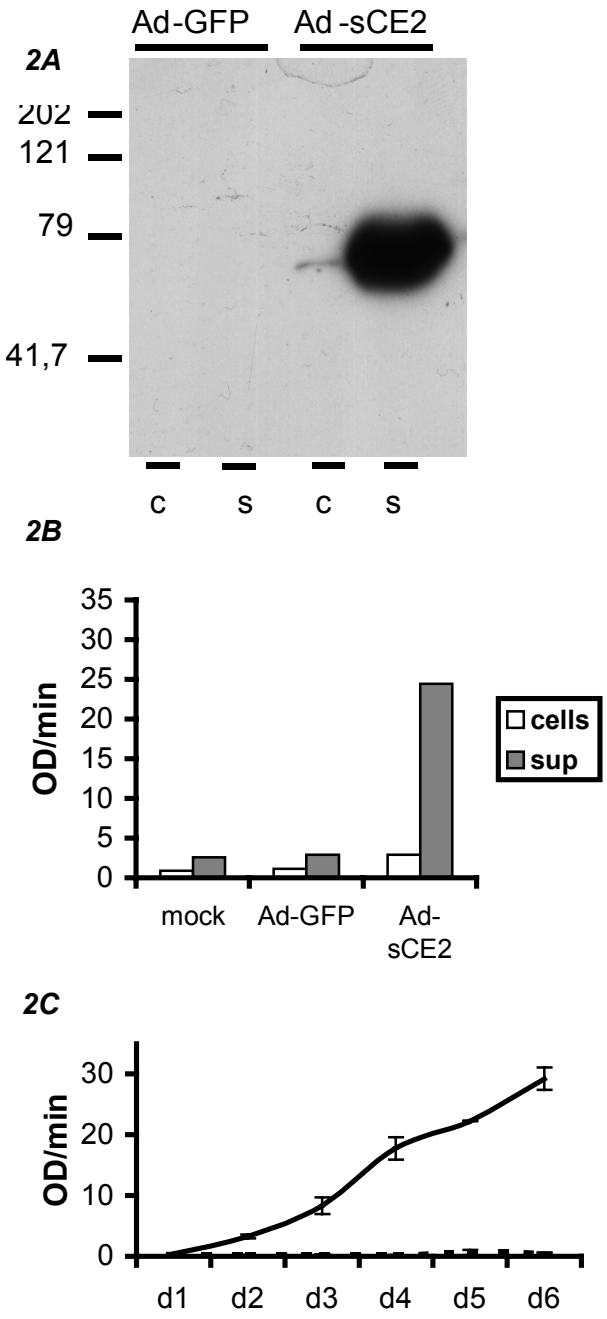
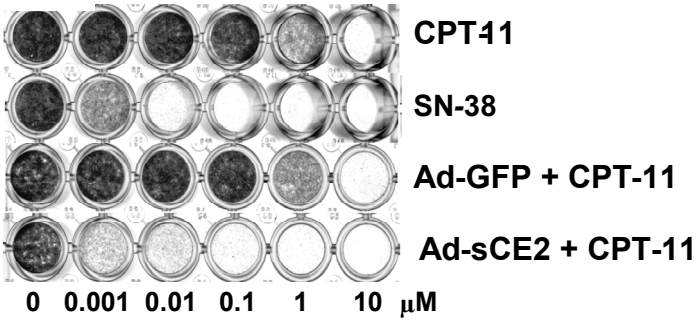



***Figure 2:*** *Characterization of Ad-sCE2 infected SaOs-2 cells by Western blot and by esterase activity assay. (A) Western blot of the cellular lysates and supernatants of SaOs-2 cells infected with Ad-sCE2 at MOI 100. As a negative control, cells were infected with Ad-GFP at MOI 100. sCE2 was detected using an antibody directed against the myc tag. In lanes 1 and 3 cellular lysates (c) and in lanes 2 and 4 supernatants (s) of SaOs-2 cells infected with Ad-GFP (lanes 1, 2) or Ad-sCE2 (lanes 3, 4) are shown. sCE2 migrated with an apparent molecular weight of 75 kDa and almost all protein was secreted. (B) CE activity in cellular lysates and supernatants of SaOs-2 cells untransduced or transduced with Ad-sCE2 or Ad-GFP at MOI 100. Samples were incubated with 1 mM pNpAc and conversion was measured over a 10-minute period. Ad-GFP infected cells did not show enhanced CE-activity in cellular lysates and culture medium compared to mock infected SaOS-2 cells, whereas Ad-sCE2 infected cells clearly expressed the CE enzyme, which was detected mainly in the supernatant. (C) CE activity in supernatants of SaOs-2 cells transduced with Ad-GFP (dotted line) or Ad-sCE2 (bold line) at MOI 100 at different time points post infection. In time, CE activity in supernatants of Ad-sCE2 transduced cells gradually increased.*

***Antiproliferative effects of CPT-11 activation in Ad-sCE2 transduced OS cells***

To show improved conversion of the prodrug CPT-11 to the toxic drug SN-38 by Ad-sCE2 encoded sCE2, OS cell lines or primary OS cells were transduced with Ad-sCE2 or Ad-GFP as a control, followed by exposure to CPT-11. Six days after transduction, surviving cells were visualized by crystal violet staining. Antiproliferative effects were quantified with the WST-1 assay. Ad-sCE2 exhibited a dose dependent sensitization to CPT-11 (not shown). Figure 3 shows the results obtained for the OS cell line SaOs-2 transduced at MOI 100. Ad-sCE2 enhanced the sensitivity of SaOs-2 cells to CPT-11 approximately 2800-fold, resulting in toxic effects comparable to treatment with SN-38 alone. Table 1 summarizes the results of the proliferation assay on all OS cell lines and primary OS cell cultures transduced at MOI 100. All OS cells tested could be sensitized to CPT-11 following infection by Ad-sCE2, ranging from 10- to 2800 fold for OS cell lines, while primary OS cells were up to 2 orders more sensitive. Interestingly, also primary cell cultures from patients resistant against cisplatin and doxorubicin treatment, i.e. OS-1a and OS-6a, were sensitized to CPT-11 by infection with Ad-sCE2. In general, in this experimental setting, where not all cells were transduced with Ad-sCE2, CPT-11 prodrug conversion was incomplete, yielding 7-70% cytotoxicity compared to SN-38 treatment.

***3A***




***3B***

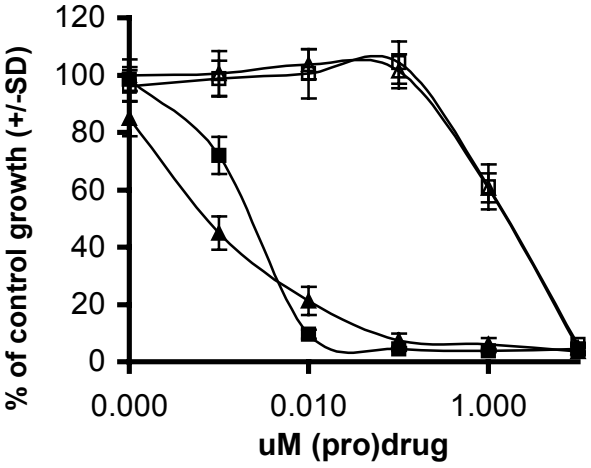



***Figure 3:*** *Cytotoxicity assay on SaOs-2 cells infected with AdsCE2 or Ad-GFP. Cell survival was determined with crystal violet staining (A) or WST-1 proliferation assay (B). (A) The SaOs-2 cells were transduced with Ad-GFP or Ad-sCE2 at MOI 100 as indicated and treated for 6 days with CPT-11 or SN-38 at the indicated concentration. Subsequently, adherent cells were stained with crystal violet and photographed. (B) Ad-GFP transduced SaOs-2 cells (□) did not show further inhibition of growth when compared to CPT-11 treated untransduced cells (Δ). Transduction with Ad-sCE2 in combination with CPT-11 (▲) resulted in toxicity comparable to exposure to SN-38 (▪). Data are presented as mean of triplicates +/- SD.*

To investigate if OS tumors could be sensitized to CPT-11 after transduction with Ad-sCE2 *in vivo*, nude mice bearing well-established subcutaneous MG-63 tumors of 200-300 $mm^3$ were treated by intratumoral injection with $1.10^9$ pfu Ad-sCE2 or PBS. Subsequently, tumors were injected on 7 consecutive days with 1.43 μg CPT-11, since this was the highest concentration of CPT-11 that did not by itself result in tumor growth inhibition as compared to PBS treatment (data not shown), or with PBS. CPT-11 was injected intratumorally since mice, in contrast to humans, have high esterase activity in their plasma [25]. Tumor growth was monitored until a volume of >2000 $mm^3$ was reached. Tumors treated with Ad-sCE2 and PBS showed a tumor growth rate (time required to reach 5 times the initial tumor volume±SD) of 4.8±1.5 days. For tumors treated with PBS and CPT-11 the tumor growth rate was 4.6±1.3 days. A significant increase in tumor growth delay was observed for the combination treatment with Ad-sCE2 plus CPT-11 (7.1±1.7 days) as compared to Ad-sCE2 alone (p=0.021) and CPT-11 alone (p=0.011) (data not shown).

Figure 4 shows in Kaplan-Meier curves the time of the tumors to reach a volume of ≥2000 $mm^3$. At a volume of 2000 $mm^3$ the animals had to be sacrificed according to animal welfare guidelines for these experiments. Curve comparison with log-rank analysis showed a significant delay in time to reach >2000 $mm^3$ tumor volume between GDEPT treated animals compared to Ad-sCE2 or CPT-11 alone (p<0.05).

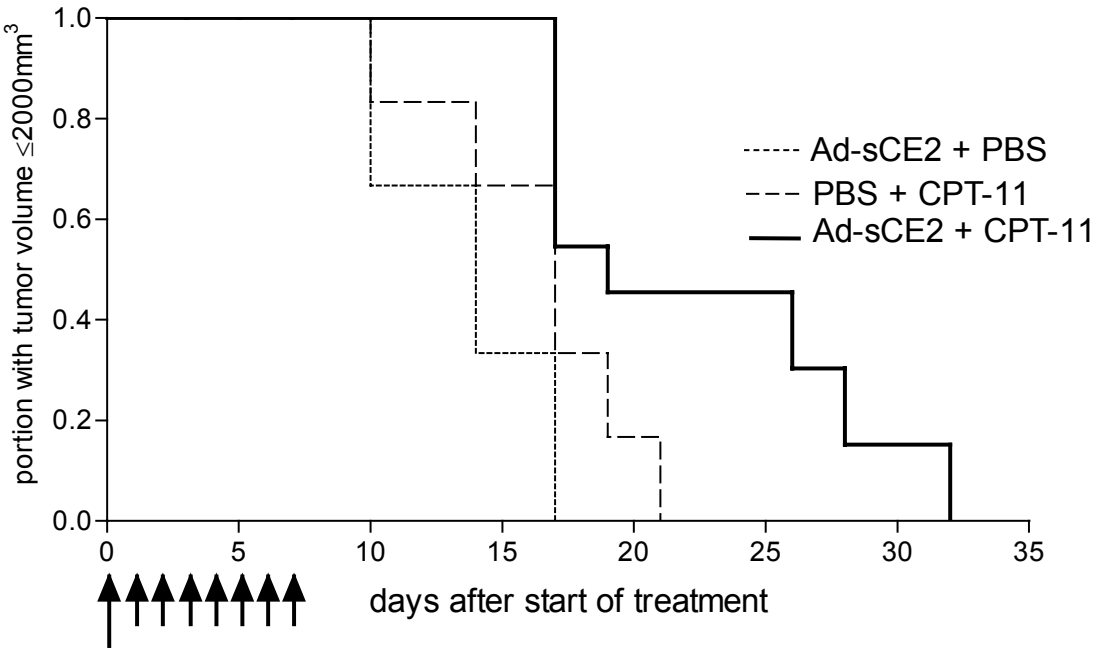



***Figure 4:*** *Kaplan-Meier curves showing the time for the tumors to reach a volume of ≥2000 $mm^3$. Curve comparison with log-rank analysis showed a significant difference in time to reach ≥2000 $mm^3$ between GDEPT treated animals compared to Ad-sCE2 or CPT-11 alone (p<0.05).*

## DISCUSSION

Despite improvement of the treatment of primary osteosarcoma (OS), the overall survival of patients varies between 50-65%. Therefore, new treatment modalities are warranted. In several studies using the enzyme-prodrug combination of herpes simplex virus thymidine kinase and ganciclovir an anti-tumor response was observed in OS models. Both local treatment and systemic treatment of lung metastasis resulted in long-term survivors [12-14]. In this study, we investigated the utility of an adenoviral vector containing the cDNA encoding a secreted form of human liver CE2, Ad-sCE2, to sensitize OS cells to the clinically approved prodrug CPT-11. OS cells transduced with Ad-sCE2 indeed secreted functional sCE2. Transduction of OS cells and primary cell cultures with Ad-sCE2 sensitized OS cells to CPT-11 *in vitro*.

Several isoforms exist from the human enzyme CE. Kojima *et al*. have described the construction of an adenovirus containing the cDNA encoding intracellular human liver CE isoform 1 (CE1) [26]. Only 3/11 cancer cell lines did show a marked (>5 fold) decrease in IC50 value after transduction and CPT-11 treatment. Humerickhouse *et al*. demonstrated that human CE2 has a higher affinity and a higher conversion velocity for CPT-11 than CE1 [27]. Based on the latter studies, we decided to use CE2 for our studies. Recently, Wierdl *et al*. described the construction of an adenoviral vector containing the cDNA encoding a secreted form of rabbit liver carboxylesterase [28], which is probably the most efficient isoform of CE [29]. Since a non-human enzyme, such as rabbit CE, might elicit an immune response in humans and repeated administration of Ad-sCE2 is anticipated in clinical applications, we preferred to express a secreted form of a human CE in our study. Therefore, it was decided to use a secreted form of human liver CE2 and as we show herein, the adenovirus expressing human *sCE2* was highly effective in GDEPT with CPT-11 for OS *in vitro*. We put particular emphasis on including primary short-term cultures. These primary OS cells were directly brought into culture after the tumor(piece) was surgically removed. Experiments were performed between passage 1 and 5 of these cultures. Therefore, these cells can be considered as reliable primary tumor cells. Although Coxsackie Adenovirus Receptor (*CAR*), expression was very low on these primary cells [19] they could still be sensitized to CPT-11 by transduction with Ad-sCE2 at a relatively high MOI. Sensitization was not complete, which could be partly attributed to differences in transduction efficiency. Interestingly, heavily pretreated primary OS cells (OS-1a and OS-6a), which were resistant to doxorubicin and cisplatin, could still be sensitized to CPT-11 by Ad-sCE2 infection.

An *in vivo* experiment in which MG-63 tumors were infected with Ad-sCE2 and treated with CPT-11 showed a significant tumor growth delay and a significant difference in the time to reach a tumor volume of >2000 $mm^3$ compared to tumors treated with either CPT-11 or Ad-sCE2 alone. Although the results differed significantly between the experimental groups, the *in vivo* treatments did not result in cures. The modest effects *in vivo* could be explained by either low transduction efficiency or a relatively low concentration of the prodrug.

The primary receptor for adenoviral entry CAR is expressed in low amounts on the cells surface of the cell line MG-63 [19]. During the *in vitro* experiments only 25% of the MG-63 cells were transduced with Ad-sCE2 as

determined by GFP expression. It is hypothesized that the MOI used to transduce MG-63 tumors *in vivo* was lower ($1.10^9$ pfu of Ad-sCE2/ 200$mm^3$ tumor). So probably, only a very small percentage of tumor cells was indeed transduced by Ad-sCE2 and this might explain the poor therapeutic effect *in vivo*. To improve this, several possibilities can be explored. First of all, the efficacy of Ad-sCE2 and CPT-11 treatment *in vivo* can perhaps be improved by redirection of the adenoviral vector to tumor antigens. We previously demonstrated that targeting an adenoviral vector towards the Epidermal Growth Factor Receptor via a bispecific antibody improved gene transfer to OS cells *in vitro* [19]. Another improvement of this approach could be the combination of enzyme prodrug therapy with conditionally replicating adenoviruses (CRAds). CRAds have shown promising preliminary results in clinical trials, especially in combination with chemotherapy. Preliminary experiments in which OS cells were transduced with Ad-sCE2 and a CRAd and treated with CPT-11 showed increased anti-cancer efficacy as compared to viral or enzyme prodrug therapy alone (unpublished results). Finally, we expect that a higher dose of CPT-11 could further enhance the efficacy of this approach. Unlike humans, however, mice have high esterase activity in their plasma, which precludes proper xenograft studies with higher doses of CPT-11 [30].

In clinical applications of GDEPT for human cancer, the adenoviral vector should be injected into the tumor lesion, while CPT-11 is given by the intravenous route. In this respect, it is questionable whether the tumor concentration of CPT-11 will be sufficient for enhanced SN-38 mediated tumor cell damage. This might perhaps be investigated in esterase deficient mice [30]. High intra-tumor concentrations of CPT-11 in humans, may however also be reached in inoperable or recurrent OS injected with Ad-sCE2 after which CPT-11 is given by intra-arterial infusion, such as in isolated limb perfusion.

Altogether, our data suggest that the combination of Ad-sCE2 and CPT-11 could be further improved and developed into a new treatment modality for OS.

## ACKNOWLEDGEMENTS

The authors thank Jeroen Mastenbroek for his expert technical assistance and Michelle de Graaf for fruitful discussions. Dinja Oosterhoff is supported by an Educational Grant from Aventis. Adhiambo Witlox is supported by The Netherlands Organization of Scientific Research (NWO-AGIKO 920-03-167). Victor van Beusechem is supported by a fellowship from the Royal Netherlands Academy of Arts and Sciences. Herbert Pinedo is supported by the Spinoza Award 1997 from the Netherlands Organization of Scientific Research.

## REFERENCES


1 Renard AJ *et al.* Osteosarcoma: oncologic and functional results. A single institutional report covering 22 years. *J Surg Oncol* 1999; **72:** 124-129.

2 Lindner NJ *et al.* Limb salvage and outcome of osteosarcoma. The University of Muenster experience. *Clin Orthop* 1999**:** 83-89.

3 Bacci G *et al.* Neoadjuvant chemotherapy for high grade osteosarcoma of the extremities: long-term results for patients treated according to the Rizzoli IOR/OS-3b protocol. *J Chemother* 2001; **13:** 93-99.

4 Bacci G *et al.* Pattern of relapse in patients with osteosarcoma of the extremities treated with neoadjuvant chemotherapy. *Eur J Cancer* 2001; **37:** 32-38.

5 Longhi A *et al.* Neoadjuvant chemotherapy for patients with synchronous multifocal osteosarcoma: results in eleven cases. *J Chemother* 2001; **13:** 324-330.

6 Kushner BH *et al.* Pilot study of topotecan and high-dose cyclophosphamide for resistant pediatric solid tumors. *Med Pediatr Oncol* 2000; **35:** 468-474.

7 Saylors RL, 3rd *et al.* Cyclophosphamide plus topotecan in children with recurrent or refractory solid tumors: a Pediatric Oncology Group phase II study. *J Clin Oncol* 2001; **19:** 3463-3469.

8 Pratesi G *et al.* Pattern of Antitumor Activity of a Novel Camptothecin, ST1481, in a Large Panel of Human Tumor Xenografts. *Clin Cancer Res* 2002; **8:** 3904-3909.

9 Satoh T *et al.* Metabolic activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1- piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. *Biol Pharm Bull* 1994; **17:** 662-664.

10 Satoh T, Hosokawa M. The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* 1998; **38:** 257-288.

11 Gupta E *et al.* Pharmacokinetic and pharmacodynamic evaluation of the topoisomerase inhibitor irinotecan in cancer patients. *J Clin Oncol* 1997; **15:** 1502-1510.

12 Ko SC *et al.* Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res* 1996; **56:** 4614-4619.

13 Cheon J *et al.* Chemogene therapy: osteocalcin promoter-based suicide gene therapy in combination with methotrexate in a murine osteosarcoma model. *Cancer Gene Ther* 1997; **4:** 359-365.

14 Shirakawa T *et al.* In vivo suppression of osteosarcoma pulmonary metastasis with intravenous osteocalcin promoter-based toxic gene therapy. *Cancer Gene Ther* 1998; **5:** 274-280.

15 Billiau A *et al.* Human interferon: mass production in a newly established cell line, MG- 63. *Antimicrob Agents Chemother* 1977; **12:** 11-15.

16 Rhim JS *et al.* Characterization of human cells transformed in vitro by N-methyl-N'- nitro-N-nitrosoguanidine. *Int J Cancer* 1977; **19:** 505-510.

17 Fogh J, Fogh JM, Orfeo T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 1977; **59:** 221-226.
18 Rochet N *et al.* Establishment, characterisation and partial cytokine expression profile of a new human osteosarcoma cell line (CAL 72). *Int J Cancer* 1999; **82:** 282-285.
19 Witlox MA *et al.* Epidermal growth factor receptor targeting enhances adenoviral vector based suicide gene therapy of osteosarcoma. *J Gene Med* 2002; **4:** 510-516.
20 He TC *et al.* A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 1998; **95:** 2509-2514.
21 Oosterhoff D *et al.* Secreted and tumour targeted human carboxylesterase for activation of irinotecan. *Br J Cancer* 2002; **87:** 659-664.
22 van Beusechem VW *et al.* Recombinant adenovirus vectors with knobless fibers for targeted gene transfer. *Gene Ther* 2000; **7:** 1940-1946.
23 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227:** 680-685.
24 Chan S *et al.* A novel tumour marker related to the c-myc oncogene product. *Mol Cell Probes* 1987; **1:** 73-82.
25 Jansen WJ *et al.* CPT-11 in human colon-cancer cell lines and xenografts: characterization of cellular sensitivity determinants. *Int J Cancer* 1997; **70:** 335-340.
26 Kojima A, Hackett NR, Ohwada A, Crystal RG. In vivo human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors. *J Clin Invest* 1998; **101:** 1789-1796.
27 Humerickhouse R *et al.* Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res* 2000; **60:** 1189-1192.
28 Wierdl M *et al.* Sensitization of Human Tumor Cells to CPT-11 via Adenoviral-mediated Delivery of a Rabbit Liver Carboxylesterase. *Cancer Res* 2001; **61:** 5078-5082.
29 Khanna R, Morton CL, Danks MK, Potter PM. Proficient metabolism of irinotecan by a human intestinal carboxylesterase. *Cancer Res* 2000; **60:** 4725-4728.
30 Morton CL *et al.* Activation of CPT-11 in mice: identification and analysis of a highly effective plasma esterase. *Cancer Res* 2000; **60:** 4206-4210.

# Chapter 5

# Gene-directed enzyme prodrug therapy with carboxylesterase enhances the anti-cancer efficacy of the conditionally replicating adenovirus Ad∆24

Dinja Oosterhoff, Herbert M. Pinedo, M. Adhiambo Witlox, Jan E. Carette, Winald R. Gerritsen and Victor W. van Beusechem

Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

Gene Therapy, advance online publication, 2005

## SUMMARY

Conditionally replicating adenoviruses (CRAds) selectively replicate in and thereby kill cancer cells. The CRAd AdΔ24 with pRb-binding deficient E1A kills cancer cells efficiently. Arming CRAds with genes encoding prodrug-converting enzymes could allow for enhanced anti-cancer efficacy by the combined effects of oncolytic replication and local prodrug activation. Here, we investigated combination treatment of human colon cancer cell lines with AdΔ24-type CRAds and Gene-Directed Enzyme Prodrug Therapy (GDEPT) using two different enzyme/prodrug systems, i.e. thymidine kinase/ganciclovir (TK/GCV) and carboxylesterase (CE)/CPT-11. On all three cell lines tested, GDEPT with TK/GCV made CRAd treatment less efficacious. In contrast, expression of a secreted form of *CE* (sCE2) combined with CPT-11 treatment markedly enhanced the efficacy of AdΔ24 virotherapy. Based on this observation, we constructed an AdΔ24 variant expressing *sCE2*. In the absence of CPT-11, this new CRAd Ad5-Δ24.E3-sCE2 was similarly effective as its parent in killing human colon cancer cells. Low concentrations of CPT-11 inhibited Ad5-Δ24.E3-sCE2 propagation. Nevertheless, CPT-11 specifically augmented the cytotoxicity of Ad5-Δ24.E3-sCE2 against all three-colon cancer cell lines. Hence, the positive contribution of sCE2/CPT-11 GDEPT to colon cancer cytotoxicity outweighed its negative influence on CRAd propagation. Therefore, CRAd-sCE2/CPT-11 combination therapy appears useful for more effective treatment of colon cancer.

## INTRODUCTION

Gene therapy with human adenoviral vectors is under investigation as an alternative approach to treat cancer. Replication-defective adenoviral gene delivery vectors have so far shown only modest anti-tumor efficacy, partly because of poor penetration capacity in solid tumor masses. To overcome this limitation, conditionally replicative adenoviruses (CRAds) were developed and explored as novel anti-cancer agents [1-3]. CRAds replicate only in cancer cells and destroy these cells through the natural process of adenoviral replication. In addition, the generated progeny viruses released from infected and lysed cancer cells may infect neighboring tumor cells. Via multiple cycles of lytic replication and lateral spread, more pronounced anti-tumor effect is accomplished. Unfortunately, however, the efficacy of CRAds as monotherapeutic agents in clinical trials has so far been limited [4,5]. Treatment modalities in which CRAd virotherapy was combined with a conventional chemotherapeutic agent such as cisplatin, 5-FU or paclitaxel were significantly more effective than treatment with either agent alone [6-8]. This synergy between CRAds and chemotherapy warranted studies into combinations of CRAds with the more selective Gene-Directed Enzyme Prodrug Therapy (GDEPT). In GDEPT, tumor cells are transduced with an expression vector encoding an enzyme that converts a non-toxic prodrug into an active drug. After systemic administration of the prodrug, this will lead to local prodrug activation at the tumor site resulting in tumor-specific toxicity. Arming CRAds with a prodrug-activating gene might thus result in augmented

anti-tumor effects similar to the combined effect of CRAds and chemotherapy, but with less systemic toxicity. In addition, the CRAd may be considered as a very potent enzyme expression vector as it is amplified in infected cells. Higher levels of prodrug-converting enzyme in the tumor should produce higher local toxic drug concentrations, thereby causing a more effective GDEPT.

Several prodrug converting enzymes, including carboxylesterase (CE) that activates the prodrug CPT-11 into the toxic drug SN-38, Herpes Simplex Virus thymidine kinase (HSV-TK) that converts the prodrug ganciclovir (GCV) into its active metabolite and nitroreductase that activates CB1954, have already been incorporated into the genome of replication competent adenoviruses [9-15]. Only in some cases, addition of the prodrug increased the oncolytic potency as expected [9,10,12]. Remarkably, in all successful studies *E1B* deleted CRAds with severely compromised replication capacity were used.

Since then, much more potent CRAds have been constructed. One of these is AdΔ24, or *dl*922-947, that contains a subtle deletion in the *E1A* region that abolishes binding of E1A to members of the Rb family of pocket proteins [16,17]. Because AdΔ24 is defective in sequestering pRb from E2F, its replication depends on E2F being released through other means. This is the case in most, if not all, cancer cells through pRb deficiency, pRb hyperphosphorylation, or pRb sequestration by cellular or viral proteins associated with malignancy. Indeed, it was demonstrated that AdΔ24 replicates in and lyses both dividing and non-dividing tumor cells with high efficiency, whereas cancer cells with restored pRb activity were resistant to the virus *in vitro* [16]. Although AdΔ24 was not entirely cancer-specific, as it was also shown to replicate in proliferating normal cell cultures and in human brain explants [17,18], the CRAd did not replicate in growth-arrested normal cell cultures [17], suggesting relative safety in normal tissues. The virus was effective against a range of cancer cells *in vitro* and *in vivo* exhibiting a potency superior to that of an *E1B* deleted CRAd and in some cancer types even to that of wild type adenovirus [17]. This led us to investigate if combining the more powerful AdΔ24 virotherapy with GDEPT using the HSV-TK/GCV or CE/CPT-11 paradigms would further increase its anti-cancer efficacy.

## MATERIAL AND METHODS

### *Cells and culture conditions*

The colon cancer cell lines SW1398, Colo205 and WiDr and the lung cancer cell line A549 were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 50 IU/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Breda, The Netherlands), at 37°C in a 5% $CO_2$ humidified atmosphere. 293 (ATCC, Manassas, VA) and 911 (Introgene, Leiden, The Netherlands) cells were maintained in DMEM supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2mM L-glutamine (Invitrogen).

***Recombinant adenoviruses***

The replication deficient adenoviral vectors Ad-sCE2 [30] and AdCMVHSV-TK [31] that express cytomegalovirus immediate early promoter-driven secreted human liver carboxylesterase-2 (*sCE2*) and *HSV-TK*, respectively, have been described previously. The conditionally replication competent adenovirus AdΔ24 containing a 24 bp deletion in the CR2 domain of the *E1A* region, resulting in selective replication in pRb mutated cells [16], has been described previously [32]. The CRAd Ad5-Δ24.E3 has the same deletion in *E1A*, but retains the entire *E3* region [19].

To construct the AdΔ24 CRAd with an expression cassette for *sCE2*, the CMV-*sCE2* expression cassette was amplified from pSTCF-sCE2 [33] by PCR with sense primer 5'-CA*GCATGC*TATGGTCGACTCTCAGTACAATCTGCTC-3' and antisense primer 5'-AAGCCATAGAGCCCACCGCATCC-3'. The resulting fragment was digested with *SphI* that cuts in the sense primer (italic) and four nucleotides adjacent to the antisense primer-annealing site and inserted into *SphI* digested pABS.4 (Microbix, Biosystems, Toronto, Canada) to generate pABS.4-sCE2. Functional expression of sCE2 was confirmed following transient transfection of the plasmid into COS-7 cells. At 2 days after transfection, supernatants were harvested and CE-activity was measured by pNpAc conversion as described previously [33]. To construct a GATEWAY compatible entry vector, pABS.4-sCE2 was digested with *Nru*I and *Kpn*I and ligated into *EcoRV*/*KpnI* digested pENTR-2B (Invitrogen) creating pENTR-sCE2. The GATEWAY system compatible adenoviral shuttle vector pEndK/DEST-R carrying the DEST cassette in the adenovirus genome between the E4 region and the right-hand ITR has been described previously [34]. The *sCE2* expression cassette of pENTR-sCE2 was transported into this pEndK/DEST-R via an LR Gateway *in vitro* recombination reaction (Invitrogen) according to the manufacturer's protocol, generating pEndK-sCE2-R containing the CMV promoter and sCE2 in the rightward orientation of the adenoviral genome.

An Ad5-Δ24.E3 derivative CRAd expressing *sCE2* was generated by homologous recombination in *E. coli* BJ5183 between Ad5-Δ24.E3 viral DNA and *EcoRV*-digested pEndK-sCE2-R to form plasmid pAd5-Δ24.E3-sCE2-R. This plasmid was digested with *PacI* to release the full-length adenoviral DNA from the plasmid backbone and was transfected into 293 cells. The CRAd Ad5-Δ24.E3-sCE2 was harvested and further propagated on A549 cells. The *E1A*-Δ24 mutation and the *sCE2* insertion were confirmed by PCR on the final products and functional plaque forming units (PFU) titers were determined by limiting-dilution plaque titration on 293 cells according to standard techniques. All infections were normalized on the basis of PFU titers.

***CE-activity in the supernatant of adenovirus infected cells***

SW1398 cells were infected with Ad5-Δ24.E3-sCE2 at a multiplicity of infection (MOI) of 0.3 or with a range of MOIs of Ad-sCE2. At different time points after infection, the supernatants were harvested and analyzed for esterase activity. Supernatant aliquots were incubated in 50 mM HEPES (pH 7.4) containing 3 mM para-nitrophenyl acetate, an esterase substrate, and conversion was monitored at 420 nm for 5 minutes on a Bio-Rad microplate

reader. Data were expressed as μmol p-nitrophenol produced per milliliter of culture media.

***Ad5-Δ24.E3-sCE2 burst size in the presence of CPT-11***

WiDr and Colo205 cells were seeded at $1.10^5$ cells/well in 24 well plates and cultured overnight. The next day, the cells were infected with Ad5-Δ24.E3-sCE2 at MOI 0.01 and cultured with or without 1 μM CPT-11. After 5 and 12 days, cells were harvested in the culture medium and subjected to three rounds of freeze/thawing to release viral particles. Titers were determined using the Adeno-X™ Rapid Titer Kit (Becton Dickinson) and a modified protocol. Briefly, 911 cells were plated ($2{,}5.10^4$ cells/well) in a 96 wells plate. The next day, cells were infected in triplicate with serially diluted Ad5-Δ24.E3-sCE2 samples. After 2 days, cells were fixed with methanol and stained with mouse anti-hexon antibody, HRP labeled rat anti-mouse antibody and DAB substrate. Hereafter, hexon-positive cells were counted in wells from at least three virus dilutions and viral titers were calculated from linear regression analyses.

***WST-1 cell viability assay on colon cancer cells treated with adenovirus and GDEPT***

Colon carcinoma cells ($1.10^4$/well) were seeded in a 96-wells microtiter plate (Greiner). After 24h, the medium was replaced with a dual virus mixture consisting of Ad-sCE2 or AdCMVHSV-TK at an MOI of 600 with AdΔ24 at a MOI of 1 or 10 or Ad5-Δ24.E3 or Ad5-Δ24.E3-sCE2 at an MOI of 1. Simultaneously, a concentration range of the prodrugs CPT-11 (Aventis, Strasbourg, France) or GCV (Roche Diagnostics, Almere, The Netherlands) was added. After six days of culture, the viability of the cells was determined by performing a WST-1 conversion assay. For that, the culture medium was removed and replaced by 100 μl 10% WST-1 (Roche Diagnostics) in culture medium. After 60-90 minutes incubation at 37°C, the $A_{450}$ was measured on a Bio-Rad microplate reader. WST-1 conversion was expressed as percentage of the conversion by uninfected, untreated control cells after subtraction of background values in the absence of cells.

***Crystal violet cytotoxicity assay on CRAd/GDEPT treated colon cancer cells***

Cells were seeded at $1.10^5$ cells/well in 24 well plates and cultured overnight. The next day, the cells were infected with Ad5-Δ24.E3-sCE2 at the indicated MOI. Hereafter, cells were cultured with or without 1 μM CPT-11, with 50% medium changes every 2-3 days. At day 11 after infection, cells were washed with PBS and fixed for 10 minutes at room temperature in 4% formaldehyde in PBS. Cells were stained using 1% crystal violet dye in 70% ethanol for 15 minutes at room temperature. After several washes with water, the culture plates were air dried and scanned on a Bio-Rad GS-690 imaging densitometer.

# RESULTS

### *TK/GCV GDEPT decreases the oncolytic potency of AdΔ24 against colon cancer cells*

Human colon cancer cell lines SW1398, WiDr and Colo205 were infected with a dual virus mixture consisting of replication deficient AdCMVHSV-TK and the CRAd AdΔ24 and treated with the prodrug GCV (Figure 1). In this system, the prodrug-converting enzyme is expressed in the context of CRAd replication. As controls, cells were treated with GCV only, infected with AdΔ24 and treated with prodrug or transduced with AdCMVHSV-TK and treated with GCV. After six days of culture, the viability of the cells was determined by WST-1 analysis.

In all three colon cancer cell lines, treatment with GCV had no effect on viability, while transduction with AdCMVHSV-TK and GCV treatment was cytotoxic at the higher concentrations of GCV, suggesting GCV activation. Infection with AdΔ24 induced dose-dependent cell death that was not affected by GCV treatment. Sensitivity of the cells to AdCMVHSV-TK/GCV treatment was not affected by co-infecting the cells with AdΔ24. However, at concentrations of GCV below 10 μM a significant reduction in AdΔ24 oncolytic toxicity on AdCMVHSV-TK transduced cells became apparent. Thus, the combination of AdΔ24 replication and HSV-TK/GCV GDEPT did not lead to enhanced colon cancer cell death. Instead, at low GCV concentrations, HSV-TK/GCV GDEPT decreased the anti-cancer effect of AdΔ24.

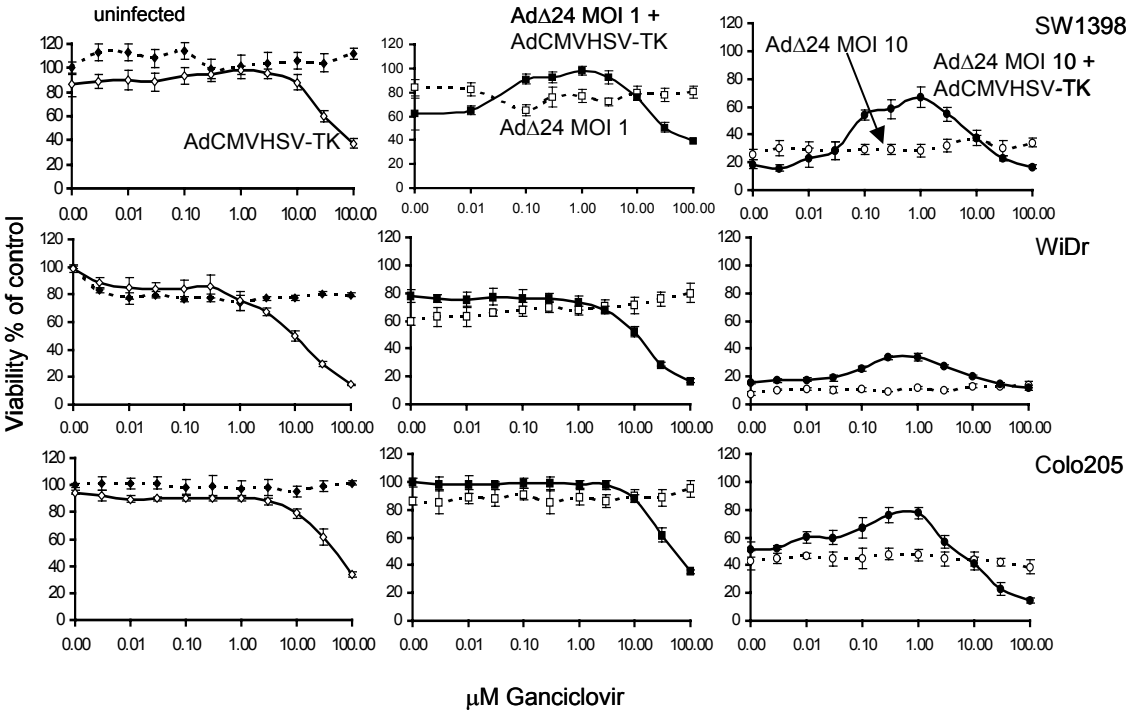



***Figure 1:*** *Exogenous expression of TK combined with GCV treatment decreases the oncolytic potency of AdΔ24 against colon cancer cells. Three colon cancer cell lines (SW1398, WiDr Colo205) were infected with AdCMVHSV-TK at MOI 600 or with AdΔ24 at MOI 1 or 10, or with dual virus mixtures and treated with GCV as indicated. After 6 days, the viability of the cells was determined with WST-1 and results are expressed as % of the untreated control cells. In all three cell lines, combination*

*treatment consisting of AdCMVHSV-TK, Ad5Δ24 and non-toxic concentrations of GCV was less effective than AdΔ24 treatment only. At higher concentrations of GCV, the efficacy of dual virus mixture was comparable to AdCMVHSV-TK/GCV only.*

### *sCE2/CPT-11 GDEPT enhances the oncolytic potency of AdΔ24 against colon cancer cells*

Similar combination experiments as described above were done with mixtures of replication deficient Ad-sCE2 expressing a secreted variant of human carboxylesterase and AdΔ24 plus CPT-11 prodrug (Figure 2). CPT-11 was toxic to the cells at concentrations above 1 μM and Ad-sCE2 sensitized the cells to CPT-11 at lower concentrations. CPT-11 treatment did not affect the oncolytic function of AdΔ24. Co-infection with AdΔ24 and Ad-sCE2 without CPT-11 treatment was as toxic as AdΔ24 infection alone. Most importantly, AdΔ24 plus Ad-sCE2/CPT-11 treatment showed a CPT-11 dose-dependent increase in cytotoxicity. At 0.1 to 1 μM CPT-11, AdΔ24 plus GDEPT was as effective or more effective than AdΔ24 virotherapy at a 10-times higher viral dose.

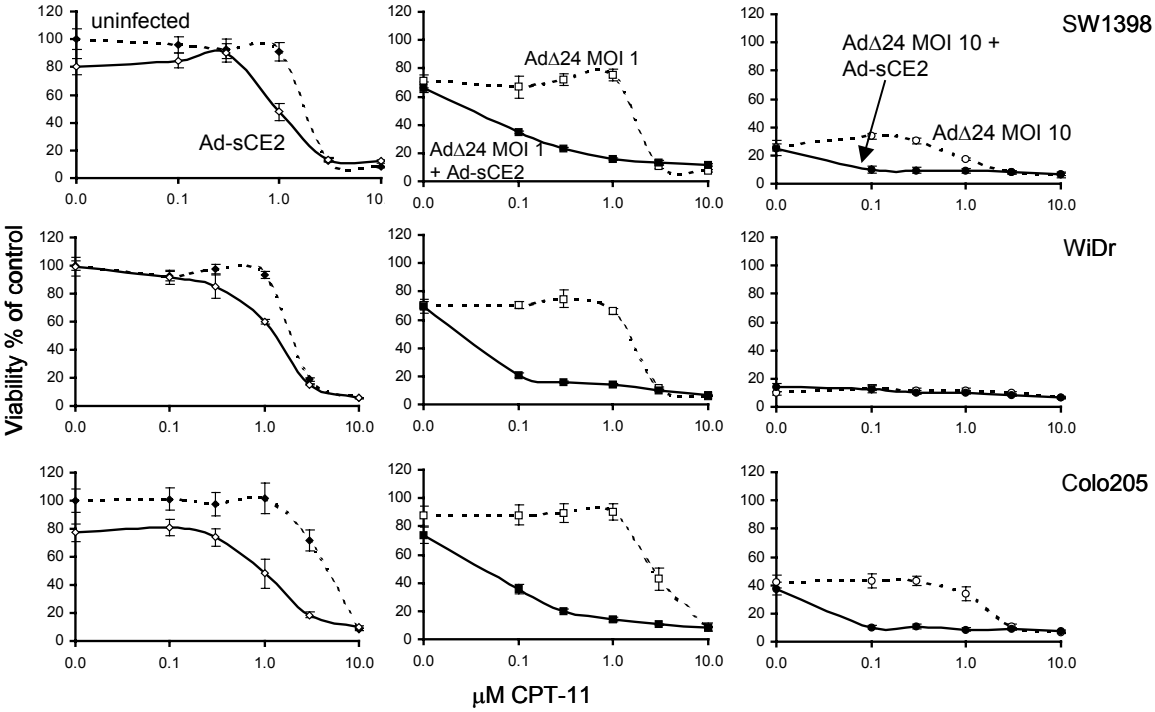



***Figure 2:*** *Exogenous expression of sCE2 in colon carcinoma cells and treatment with non-toxic concentrations of CPT-11 enhances the oncolytic efficacy of AdΔ24. SW1398, WiDr and Colo205 were infected with Ad-sCE2 at MOI 600 or with AdΔ24 at MOI 1 or 10, or with dual virus mixtures and treated with CPT-11 as indicated. The viability of the cells was determined after 6 days, as described in the legend to figure 1. AdΔ24-infected cells expressing sCE2 were sensitized to CPT-11 treatment.*

Next, we conducted similar combination experiments with sCE2/CPT-11 and Ad5-Δ24.E3. In contrast to AdΔ24, Ad5-Δ24.E3 contains an intact *E3* region, which encodes the adenoviral death protein that promotes cytolysis [19].

In addition, E3 proteins contribute to virotherapy efficacy *in vivo* [20]. Consequently, Ad5-Δ24.E3 is more potent than AdΔ24. Combination experiments showed that the efficacy of Ad5-Δ24.E3 could also be further improved by expression of sCE2 and treatment with non-toxic doses of CPT-11 (Figure 3). Hence, in contrast to HSV-TK/GCV GDEPT, sCE2/CPT-11 GDEPT enhanced the oncolytic potency of strong AdΔ24-type CRAds.

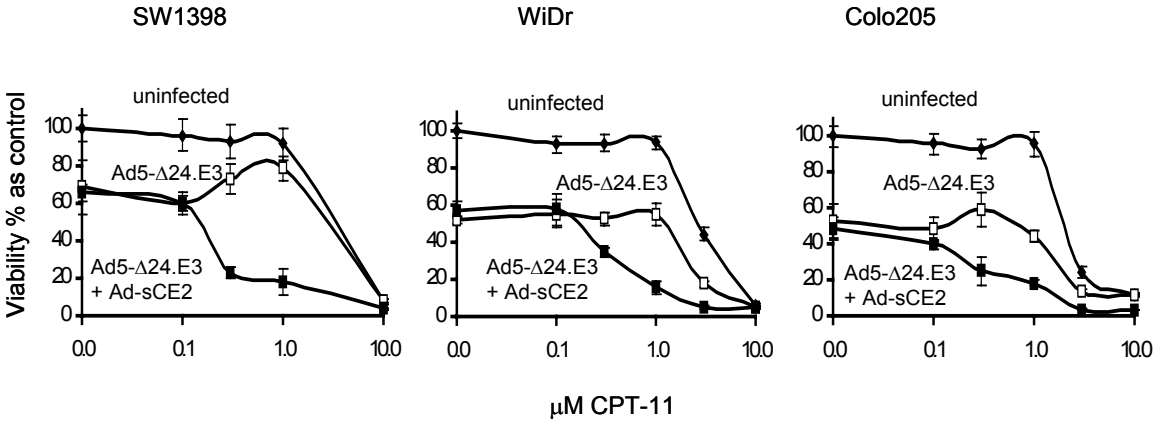



***Figure 3:*** *sCE2/CPT-11 treatment enhances the oncolytic efficacy of the CRAd Ad5-Δ24.E3 against colon carcinoma cells. SW1398, WiDr and Colo205 were infected with Ad-sCE2 at MOI 600 or with Ad5-Δ24.E3 at MOI 1, or with dual virus mixtures and treated with CPT-11 as indicated. The viability of the cells was determined after 6 days, as described in the legend to figure 1. Cells expressing sCE2 and infected with Ad5-Δ24.E3 were sensitized to CPT-11.*

### *Construction and characterization of sCE2 expressing CRAd Ad5-Δ24.E3-sCE2*

Based on the observations described above, we decided to construct an Ad5-Δ24.E3 derived CRAd expressing *sCE2*, which is schematically shown in figure 4A. Insertion of the *sCE2* expression cassette into the adenoviral genome, creating a virus with an approximate 108% wild type genome size, did not affect propagation efficiency (not shown). Proper expression of *sCE2* by the new CRAd Ad5-Δ24.E3-sCE2 was tested by infecting SW1398 cells at low MOI and measuring CE-activity in the supernatant of infected cells at different time points after infection. As expected, extracellular CE-activity increased in time, as the CRAd replicated and spread in the culture (Figure 4B). In the same experiment, SW1398 cells were transduced with the replication deficient adenovirus Ad-sCE2 at various MOIs. As can be seen in figure 4C, a more than 300-fold higher MOI of the replication deficient vector was needed to obtain comparable CE-activity in the supernatant at day 5 after transduction.

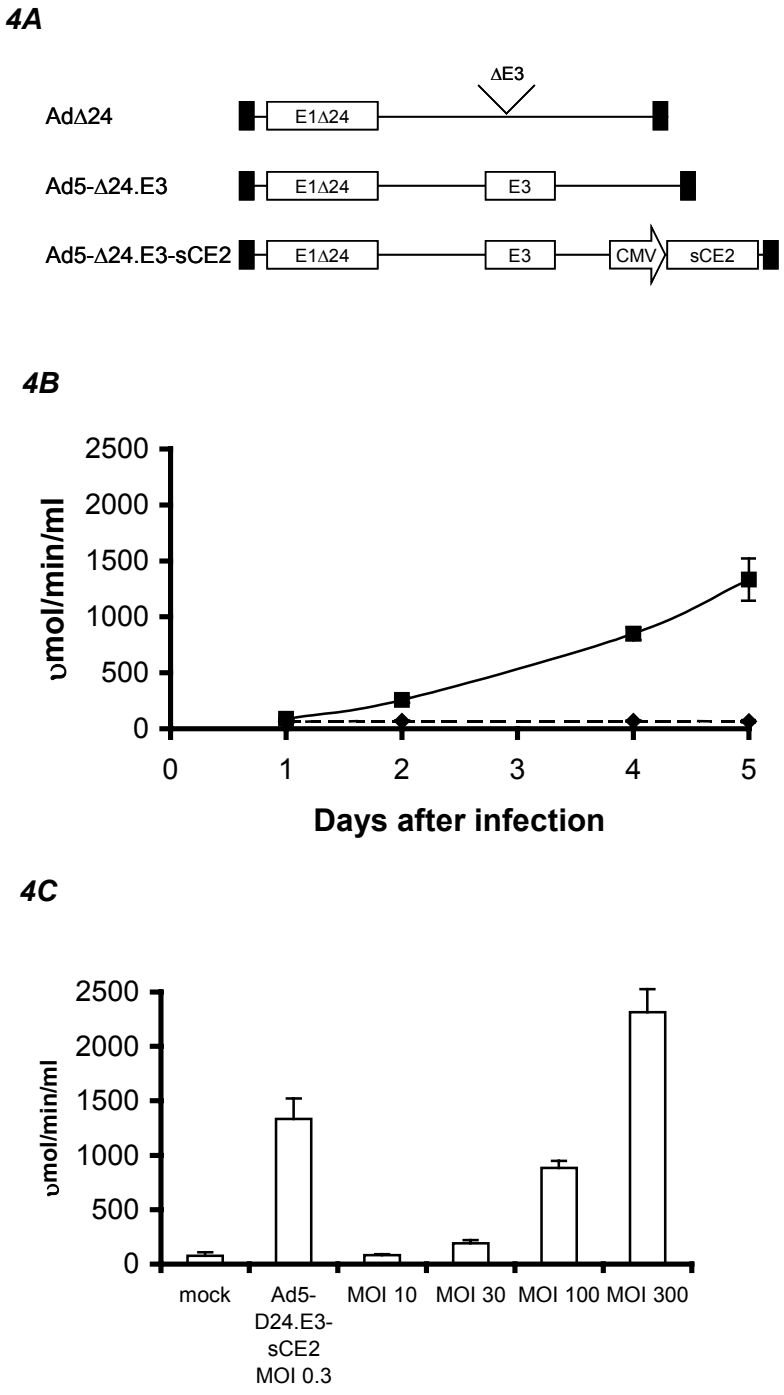



***Figure 4:*** *Construction and characterization of Ad5-Δ24.E3-sCE2. (A) Schematic presentation of the conditionally replicating adenoviruses used in this study. (B) SW1398 cells were infected with Ad5-Δ24.E3-sCE2 at MOI 0.3 (squares) or mock treated (diamonds) and the CE-activity in the medium was determined at days 1, 2, 4 and 5 after infection. (C) Comparison of the CE-activity in the medium 5 days after infection of SW1398 cells with Ad5-Δ24.E3-sCE2 at MOI 0.3 or with replication deficient Ad-sCE2 at various MOI. Data represent mean values +/- SD of an experiment performed in quintuples.*

To analyze the effect of sCE2/CPT-11 enzyme prodrug therapy on viral replication, Colo205 and WiDr cells were infected with Ad5-Δ24.E3-sCE2 at low MOI and the viral titer was determined 5 and 12 days later. As can be seen in figure 5, CPT-11 treatment decreased the viral output from Ad5-Δ24.E3-sCE2 infected cells up to 300-fold. Virus expansion from day 5 to 12 post-infection was decreased by 10-fold. Thus, sCE2/CPT-11 GDEPT inhibited CRAd propagation.

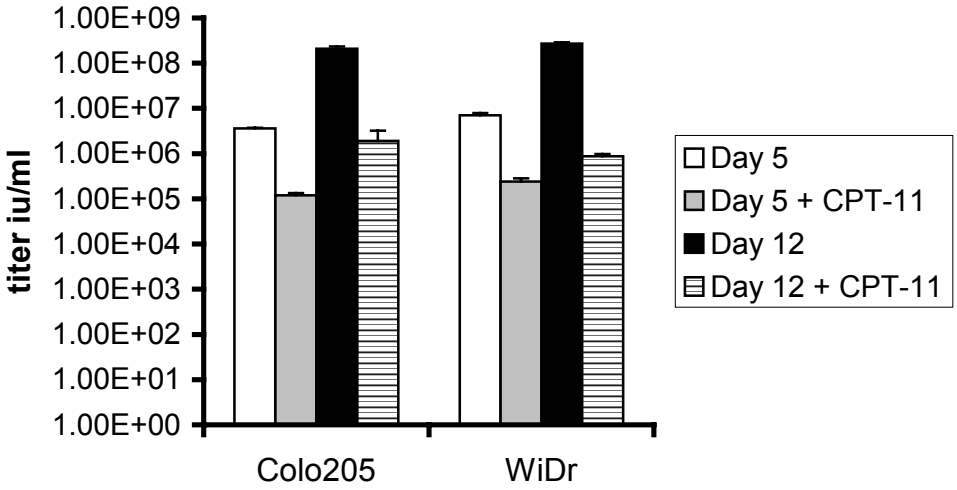



***Figure 5:*** *The replication of Ad5-Δ24.E3-sCE2 is inhibited by CPT-11 treatment. Colo205 and WiDr cells were infected with Ad5-Δ24.E3-sCE2 at MOI 0.01 and treated with CPT-11. At days 5 and 12 after infection the viral titer was determined.*

### *Ad5-Δ24.E3-sCE2 exhibits enhanced cytotoxicity against colon cancer cell lines when combined with CPT-11 treatment*

To determine the cytotoxic potency of Ad5-Δ24.E3-sCE2, human colon cancer cell lines were infected with Ad5-Δ24.E3-sCE2 or with the parent control virus Ad5-Δ24.E3 and treated with CPT-11. After six days, viability of the cells was determined by WST-1 assay. Figure 6 shows that in the absence of CPT-11, *sCE2* transgene expression did not change CRAd oncolytic potency on Colo205 and SW1398 cells and only slightly enhanced killing of WiDr cells. Most importantly, Ad5-Δ24.E3-sCE2, but not Ad5-Δ24.E3, sensitized colon cancer cells to CPT-11. At an MOI at which Ad5-Δ24.E3-sCE2 and its parent Ad5-Δ24.E3 were only mildly toxic, addition of a non-toxic concentration of 1 μM CPT-11 killed 60-80% of Ad5-Δ24.E3-sCE2-infected colon cancer cells.

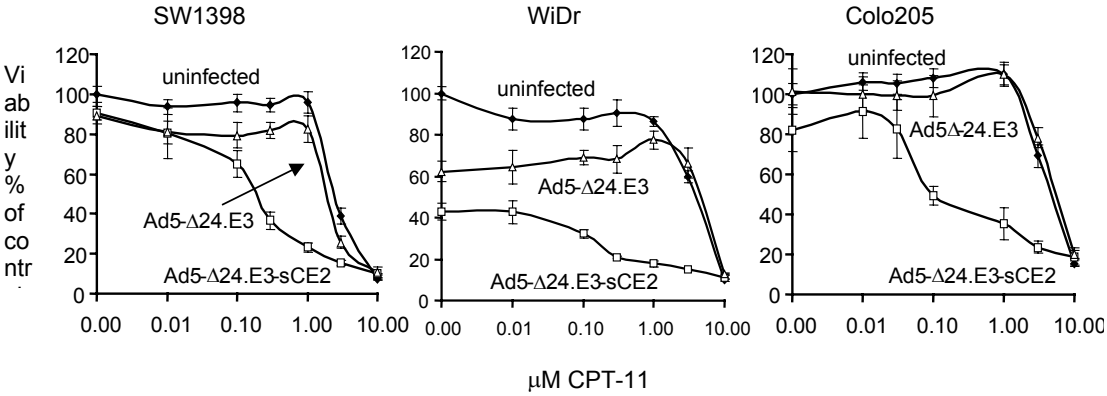



***Figure 6:*** *CPT-11 increases the anti-tumor effect of Ad5-Δ24.E3-sCE2 against colon cancer cells. SW1398, WiDr and Colo205 cells were infected with Ad5-Δ24.E3 or Ad5-Δ24.E3-sCE2 at MOI 1 and treated with CPT-11. At non-toxic concentrations, CPT-11 enhanced the oncolytic effect of Ad5-Δ24.E3-sCE2, but not of the parental virus Ad5-Δ24.E3.*

To study oncolytic replication of the new CRAd Ad5-Δ24.E3-sCE2 in the continued presence of CPT-11, colon cancer cell lines were infected with Ad5-Δ24.E3-sCE2 at low MOI and cultured in the presence of a non-toxic concentration of CPT-11 or in plain medium. At several days post infection, cells that had survived the treatment were stained with crystal violet. This revealed a progressive destruction of all monolayers by replicating Ad5-Δ24.E3-sCE2. After 11 days, surviving cells were stained with crystal violet. Figure 7 shows more prominent plaques indicative of lytic replication in the absence of CPT-11, suggesting CRAd propagation inhibition by sCE2/CPT-11 GDEPT. However, it can also be seen that Ad5-Δ24.E3-sCE2 eradicated monolayers of all three colon cancer cell lines more effectively in the presence of a non-toxic dose of CPT-11. Hence, despite the inhibitory influence of sCE2/CPT-11 GDEPT on CRAd propagation, CRAd/GDEPT combination therapy was still more effective than either treatment alone.

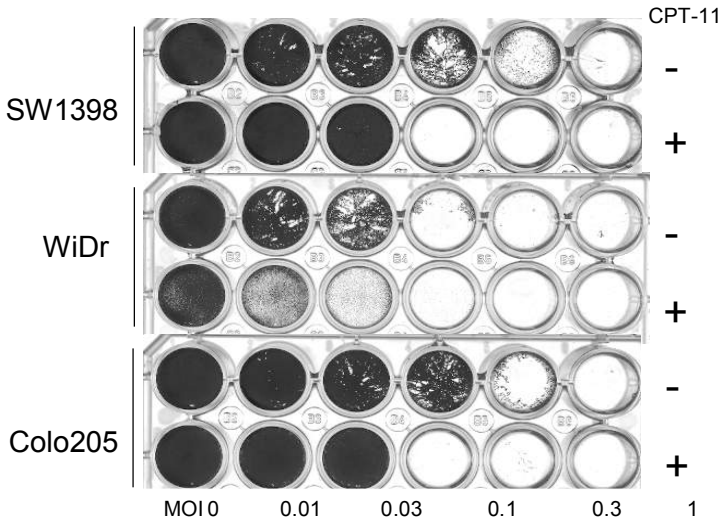



***Figure 7:*** *Ad5-Δ24.E3-sCE2 replicates in colon cancer cells in the continuous presence of CPT-11. SW1398, Colo205 and WiDr cells were infected with Ad5-Δ24.E3-sCE2 at the indicated MOI and cultured with or without 1 μM CPT-11. At day 11 after infection, attached cells were stained with crystal violet. Combination treatment was more effective than virotherapy alone.*

## DISCUSSION

Combining CRAd virotherapy with GDEPT is under evaluation for effective anti-cancer treatment. Previously, HSV-TK/GCV GDEPT has been tested in the context of *E1B-55kD* deleted CRAds [9,10] or wild type adenovirus [11,13,14]. In general, these studies showed that HSV-TK/GDEPT primarily enhanced the oncolytic potency of severely attenuated *E1B-55kD* deleted CRAds, whereas the anti-tumor efficacy of more potent wild type viruses decreased when combined with TK/GCV GDEPT. Interestingly, Nanda *et al*. found that although TK/GCV treatment completely abrogated wild type adenovirus replication when administered concomitantly, it significantly enhanced the oncolytic potency of wild type adenovirus when GCV administration was started one or two days after virus injection [21]. Virotherapy has also been combined with HSV-TK/GCV and cytosine deaminase (CD)/5-FC double GDEPT. Freytag *et al*. showed that an *E1B-55kD* deleted CRAd expressing a fusion gene consisting of *CD* and *TK* was quite effective in killing cancer cells *in vitro* when combined with GCV or 5-FC, which effect could be further augmented by irradiation [22]. Interestingly, these effects occurred at prodrug

concentrations that inhibited adenovirus replication, suggesting that the CRAd acted merely as an effective prodrug-converting gene transfer vector. Furthermore, combination therapy of colon cancer cells with an *E1B-55kD* deleted CRAd expressing the enzyme *nitroreductase* and the prodrug CB1954 also demonstrated that prodrug treatment can inhibit viral replication [15]. Recently, Stubdal *et al*. incorporated the rabbit *CE* gene into an *E1B* deleted CRAd [12]. *In vitro*, this virus exhibited increased toxicity on a colon carcinoma cell line in the presence of CPT-11 compared to control virus. However, treatment with CPT-11 did not enhance survival of mice carrying CRAd-CE injected subcutaneous colon cancer xenografts. In the aggregate, previous results suggest that arming CRAds with transgenes for GDEPT can improve their oncolytic potential. However, careful evaluation of CRAd efficacy enhancing versus abrogating activities of GDEPT is needed. Apparently, in the context of virotherapy, there is a delicate balance between GDEPT induced cancer cell death and GDEPT mediated impairment of viral replication.

Since the aim of arming CRAds with therapeutic genes such as those encoding prodrug-converting enzymes is to augment their anti-cancer potency, studies into combining CRAds and GDEPT are most useful if they are performed with the most powerful types of CRAds. Therefore, we studied whether the CRAd AdΔ24, which is much more potent than *E1B*-deleted CRAds [17], could be further improved by combinations with HSV-TK/GCV or sCE2/CPT-11 GDEPT. To this end, we used an *in vitro* adenovirus-mixing model where cells are transduced with AdΔ24 and a replication deficient adenovirus expressing *TK* or *sCE2*. Using this model, we could select a suitable CRAd-GDEPT combination prior to actually constructing the CRAd expressing the prodrug-converting enzyme. Hence, the mixing model could proof useful for quick evaluation of other CRAd-GDEPT systems as well.

We found that HSV-TK/GCV GDEPT decreased treatment efficacy, which was in line with earlier studies showing that HSV-TK/GCV GDEPT did also not work in combination with wild type adenovirus [11,13,14]. It can thus be concluded that GCV/TK GDEPT is not suitable to improve the efficacy of potent CRAds. In contrast, expression of *sCE2* in the context of AdΔ24 replication combined with CPT-11 treatment increased the efficacy of virotherapy. This was also true for Ad5-Δ24.E3, a more potent AdΔ24 variant that retains the *E3* region [19]. Therefore, we constructed Ad5-Δ24.E3-sCE2, a derivative of Ad5-Δ24.E3 expressing *sCE2*. This new CRAd differs materially from the previously described *E1B* deleted *CE*-expressing CRAd ONYX-713 [12] in that Ad5-Δ24.E3-sCE2 is a derivative from the more potent CRAd AdΔ24 and that it encodes a secreted form of human *CE-2* instead of an intracellular form of rabbit *CE*.

Ad5-Δ24.E3-sCE2 replicated efficiently in human colon cancer cells and expressed high levels of functional sCE2 protein. In the presence of CPT-11, however, Ad5-Δ24.E3-sCE2 viral propagation rate was markedly decreased. This observation can perhaps be explained by the fact that the active drug SN-38 that is produced through sCE2-mediated conversion of CPT-11 inhibits topoisomerase I, which has been demonstrated essential for viral replication [23,24,25]. Nevertheless, sCE2/GCV GDEPT enhanced the efficacy of Ad5-Δ24.E3 against colon cancer cells. This increased efficacy of

combination treatment is probably due to a strong bystander effect of the secreted prodrug-converting enzyme causing toxicity of extracellularly converted CPT-11 to uninfected neighboring cells. Such a bystander effect will contribute to the observed virus propagation inhibition, as cells already affected by SN-38 prior to Ad5-Δ24.E3-sCE2 infection will not support CRAd replication. Therefore, treatment regimens might perhaps be optimized by delayed administration of CPT-11.

Several further improvements to CRAd-sCE2/CPT-11 treatment can be foreseen. First, double *E1A* mutant CRAds have been described with a more stringent replication pattern than AdΔ24 [26,27]. The selectivity of the *CE*-expressing CRAd might thus be improved by using such viruses as backbones. Second, selective expression of the transgene in cancer cells could contribute to diminishing GDEPT cytotoxicity to non-malignant cells. Such can be accomplished by driving the expression of the prodrug-converting enzyme by a cancer cell specific promoter or by the endogenous adenovirus major late promoter [28]. Third, leakage of sCE2 from the tumor, potentially causing systemic toxicity, can be prevented by constructing a fusion protein consisting of sCE2 and an scFv antibody directed to a tumor antigen [29].

In conclusion, we have demonstrated that, in contrast to HSV-TK/GCV GDEPT, sCE2/CPT-11 GDEPT can be used to augment the anticancer potency of already very effective AdΔ24-type CRAds. This warrants further evaluation of CRAd plus sCE2/CPT-11 GDEPT strategies for colorectal cancer.

## REFERENCES


1 Haviv YS, Curiel DT. Engineering regulatory elements for conditionally-replicative adeno-viruses. *Curr Gene Ther 2003*; **3:** 357-385.

2 Post LE. Selectively replicating adenoviruses for cancer therapy: an update on clinical development. *Curr Opin Investig Drugs 2002*; **3:** 1768-1772.

3 Oosterhoff D, van Beusechem VW. Conditionally replicating adenoviruses as anticancer agents and ways to improve their efficacy. *J Exp Ther Oncol 2004*; **4:** 37-57.

4 Nemunaitis J *et al.* Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. *Gene Ther 2001*; **8:** 746-759.

5 DeWeese TL *et al.* A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res 2001*; **61:** 7464-7472.

6 Heise C *et al.* ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med 1997*; **3:** 639-645.

7 Heise C, Lemmon M, Kirn D. Efficacy with a replication-selective adenovirus plus cisplatin-based chemotherapy: dependence on sequencing but not p53 functional status or route of administration. *Clin Cancer Res 2000*; **6:** 4908-4914.

8 You L, Yang CT, Jablons DM. ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res 2000*; **60:** 1009-1013.
9 Wildner O *et al.* Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. *Gene Ther 1999*; **6:** 57-62.
10 Wildner O, Blaese RM, Morris JC. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. *Cancer Res 1999*; **59:** 410-413.
11 Wildner O, Morris JC. The role of the E1B 55 kDa gene product in oncolytic adenoviral vectors expressing herpes simplex virus-tk: assessment of antitumor efficacy and toxicity. *Cancer Res 2000*; **60:** 4167-4174.
12 Stubdal H *et al.* A prodrug strategy using ONYX-015-based replicating adenoviruses to deliver rabbit carboxylesterase to tumor cells for conversion of CPT-11 to SN-38. *Cancer Res 2003*; **63:** 6900-6908.
13 Morris JC, Wildner O. Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-tk. *Mol Ther 2000*; **1:** 56-62.
14 Lambright ES *et al.* Inclusion of the herpes simplex thymidine kinase gene in a replicating adenovirus does not augment antitumor efficacy. *Gene Ther 2001*; **8:** 946-953.
15 Chen MJ *et al.* Enhanced efficacy of Escherichia coli nitroreductase/CB1954 prodrug activation gene therapy using an E1B-55K-deleted oncolytic adenovirus vector. *Gene Ther 2004*; **11:** 1126-1136.
16 Fueyo J *et al.* A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene 2000*; **19:** 2-12.
17 Heise C *et al.* An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat Med 2000*; **6:** 1134-1139.
18 Geoerger B *et al.* Oncolytic activity of p53-expressing conditionally replicative adenovirus AdDelta24-p53 against human malignant glioma. *Cancer Res 2004*; **64:** 5753-5759.
19 Suzuki K, Alemany R, Yamamoto M, Curiel DT. The presence of the adenovirus E3 region improves the oncolytic potency of conditionally replicative adenoviruses. *Clin Cancer Res 2002*; **8:** 3348-3359.
20 Wang Y *et al.* E3 gene manipulations affect oncolytic adenovirus activity in immunocompetent tumor models. *Nat Biotechnol 2003*; **21:** 1328-1335.
21 Nanda D *et al.* Treatment of malignant gliomas with a replicating adenoviral vector expressing herpes simplex virus-thymidine kinase. *Cancer Res 2001*; **61:** 8743-8750.
22 Freytag SO *et al.* A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther 1998*; **9:** 1323-1333.
23 Nagata K, Guggenheimer RA, Hurwitz J. Adenovirus DNA replication in vitro: synthesis of full-length DNA with purified proteins. *Proc Natl Acad Sci U S A 1983*; **80:** 4266-4270.

24 Schaack J, Schedl P, Shenk T. Topoisomerase I and II cleavage of adenovirus DNA in vivo: both topoisomerase activities appear to be required for adenovirus DNA replication. *J Virol 1990*; **64:** 78-85.
25 Wong ML, Hsu MT. Involvement of topoisomerases in replication, transcription, and packaging of the linear adenovirus genome. *J Virol 1990*; **64:** 691-699.
26 Howe JA *et al.* Evaluation of E1-mutant adenoviruses as conditionally replicating agents for cancer therapy. *Mol Ther 2000*; **2:** 485-495.
27 Balague C *et al.* Human papillomavirus E6E7-mediated adenovirus cell killing: selectivity of mutant adenovirus replication in organotypic cultures of human keratinocytes. *J Virol 2001*; **75:** 7602-7611.
28 Fuerer C, Iggo R. 5-Fluorocytosine increases the toxicity of Wnt-targeting replicating adenoviruses that express cytosine deaminase as a late gene. *Gene Ther 2004*; **11:** 142-151.
29 Oosterhoff D *et al.* Adenoviral vector mediated expression of a gene encoding secreted, EpCAM targeted carboxylesterase-2 sensitises colon cancer spheroids to CPT-11. *Br J Cancer 2004*; in press.
30 Oosterhoff D *et al.* Gene-directed enzyme prodrug therapy for osteosarcoma: sensitization to CPT-11 in vitro and in vivo by adenoviral delivery of a gene encoding secreted carboxylesterase-2. *Mol Cancer Ther 2003*; **2:** 765-771.
31 Rosenfeld ME *et al.* Adenoviral-mediated delivery of the herpes simplex virus thymidine kinase gene selectively sensitizes human ovarian carcinoma cells to ganciclovir. *Clin Cancer Res 1995*; **1:** 1571-1580.
32 van Beusechem VW *et al.* Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer Res 2002*; **62:** 6165-6171.
33 Oosterhoff D *et al.* Secreted and tumour targeted human carboxylesterase for activation of irinotecan. *Br J Cancer 2002*; **87:** 659-664.
34 Carette JE *et al.* Conditionally replicating adenoviruses expressing short hairpin RNAs silence the expression of a target gene in cancer cells. *Cancer Res 2004*; **64:** 2663-2667.

# Chapter 6

# The conditionally replicative adenovirus AdΔ24-p53 and oxaliplatin act synergistically against colon carcinoma

Dinja Oosterhoff, Epie Boven, D.C. Jeroen Mastenbroek, Winald R. Gerritsen, Herbert M. Pinedo and Victor W. van Beusechem

Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands

## SUMMARY

Standard treatment modalities for colorectal cancer include surgery and chemotherapy. Oxaliplatin is a novel platinum-based drug and is approved for the treatment of colorectal cancer. However, the success of chemotherapy as single treatment modality is still limited and therefore novel combination treatment modalities are warranted. Conditionally replicative adenoviruses (CRAds) selectively replicate in and thereby kill cancer cells. CRAds have shown limited efficacy as single modality treatment in clinical trials. Combining CRAds with conventional chemotherapy resulted in synergistic efficacy in a range of tumors. Here, we compared the anti tumor efficacy of two different CRAds on colon cancer cells, i.e., AdΔ24 that selectively replicates in cancer cells with a defective pRb pathway and its *p53*-expressing derivative AdΔ24-p53. Furthermore, we investigated combination treatment of these CRAds with oxaliplatin. We found that AdΔ24-p53 was more oncolytic than AdΔ24 against all tested colon cancer cell lines, with AdΔ24-p53 achieving comparable effects at a 4-20-fold lower dose. In combination with a sub-toxic concentration oxaliplatin, AdΔ24-p53 exhibited a supra-additive killing potency. Similar synergistic effects were observed following combination treatment of oxaliplatin with AdΔ24 or with the *p53*-expressing replication deficient adenoviral vector Adwtp53. This suggested that the synergy between AdΔ24-p53 and oxaliplatin is most likely the result of two complementing mechanisms, i.e., chemosensitization by p53 gene therapy and by CRAd replication. Combination therapy consisting of AdΔ24-p53 virotherapy and oxaliplatin chemotherapy thus appears very promising for the treatment of colorectal cancer.

## INTRODUCTION

Colon cancer is the second leading cause of cancer deaths in Western countries. Between 20% and 50% of patients with colorectal cancer will die within five years of diagnosis [1], usually as a result of extensive metastatic disease. At the time of diagnosis, 20% of patients have metastases in the liver, the predominant metastatic site for colorectal cancer. Standard treatment for colorectal cancer includes surgery and chemotherapy. Platinum-based drugs are among the most active anticancer agents and have been widely used in the treatment of colon carcinoma. Over the last 30 years, a large number of platinum analogues has been synthesized to enlarge the spectrum of activity, overcome cellular resistance and/or reduce the toxicity of both first and second generation platinum drugs, like cisplatin and carboplatin [2]. Of these platinum analogues, compounds containing a diaminocyclohexane carrier ligand, such as oxaliplatin [3-5], have consistently demonstrated antitumor activity in cell lines with acquired cisplatin resistance and appear to be active in tumor types that are intrinsically resistant to cisplatin and carboplatin [6-8]. Like cisplatin, oxaliplatin forms platinum-DNA adducts, but these adducts are bulkier and induce a greater deformation of the DNA structure [9,10]. Consequently, oxaliplatin is more effective in inhibiting DNA synthesis [11,12]. Despite improvements in the efficacy of chemotherapy for

colorectal cancer, its success is still limited by several drawbacks, including insufficient drug concentrations in the tumor, systemic toxicity, lack of selectivity for tumor cells over normal cells, and the appearance of drug-resistant tumor cells. Therefore, there is a need for more effective treatment modalities. Gene therapy approaches for colorectal cancer using conditionally replicative adenoviruses (CRAds), designed to selectively replicate in tumor cells and to destroy these cells by inducing lysis (reviewed in [13,14]), have demonstrated promising results in preclinical models. However, the primary conclusion from cancer gene therapy trials with replication deficient adenoviral vectors is that low efficiency of gene transfer to tumor cells remains the key factor limiting clinical efficacy. The release of viral progeny from lysed tumor cells offers the potential to amplify CRAds at the site of the tumor and to achieve lateral spread to neighboring cells. The CRAd AdΔ24, or Ad*dl*922-947, is a potent cytolytic agent that has a deletion of 24 bp in the adenovirus *E1A* gene, resulting in an E1A protein that cannot bind to the cellular retinoblastoma protein (pRb) [15,16]. pRb modulates the cell cycle by regulating progression from G1 into S-phase. Wild type E1A binds to pRb thereby releasing the transcription factor E2F that can subsequently activate cell cycle regulatory genes. This allows S-phase entry and virus replication in otherwise quiescent cells. Because AdΔ24 is defective in sequestering pRb from E2F, its replication depends on E2F being released via other mechanisms. In most, if not all, cancer cells this is the case through pRb deficiency, pRb hyperphosphorylation, or pRb sequestration by cellular or viral proteins associated with malignancy [17]. Therefore, AdΔ24 should be unable to replicate in normal tissues, whereas its replication is not hampered in tumor cells. Previously, we constructed a derivative of AdΔ24, AdΔ24-p53 that contains a constitutive expression cassette for the tumor suppressor protein p53. On most tested cancer cell lines, AdΔ24-p53 exhibited enhanced oncolytic potency compared to AdΔ24 [18]. The superior anti-cancer efficacy of AdΔ24-p53 was further corroborated on primary heterogeneous glioma specimens and in xenograft animal models [19,20].

Multimodality treatments are usually more effective against cancer than single treatments. Several studies have already been performed combining virotherapy with chemotherapy. In lung cancer cell lines and primary cultures as well as in prostate cancer cells, CRAds worked synergistically with the platinum-based anticancer agent paclitaxel [21,22].

Therefore, in this study we tested the efficacy of the strong CRAd AdΔ24-p53 in combination with the improved platinum-based drug oxaliplatin against colon carcinoma cell lines.

## MATERIALS AND METHODS

### *Cells and culture conditions*

The colon cancer cell lines SW1398, Colo205 and WiDr were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 50 IU/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Breda, The Netherlands), at 37°C in a 5% $CO_2$ humidified atmosphere.

***Recombinant adenoviruses***

AdΔ24 and AdΔ24-p53 [18] are conditionally replication competent adenoviruses that both contain a 24 bp deletion in the *E1A* region, resulting in selective replication in tumor cells. Both viruses lack the *E3* region. AdΔ24-p53 carries an SV40 early promoter driven *p53* expression cassette in place of the *E3* region. The *E1*/*E3* deleted replication deficient adenoviral vector Adwtp53 [23] carries the same expression cassette in place of *E1*. The replication deficient adenovirus was propagated on the permissive cell line 293, whereas the CRAds were propagated on the lung cancer cell line A549. All viral aliquots were stored at -80°C until use. Adenovirus titers (pfu/ml) were determined by limiting dilution assay on 293 cells.

***In vitro cytotoxicity assays***

Colon carcinoma cells ($1.10^4$/well) were plated in a 96-wells microtiter plate (Greiner). After 24h, cells were transduced with various multiplicities of infection (MOI) of AdΔ24 or AdΔ24-p53 in 100 μl culture medium as indicated. Alternatively, cells were transduced with the replication deficient adenovirus Adwtp53 at an MOI of 300 pfu/cell. For combination experiments with chemotherapy, different concentrations of oxaliplatin were administered to the cells simultaneously with the virus. After 6 or 7 days, cell viability was determined by WST-1 (Roche Diagnostics, Mannheim, Germany) conversion at 37°C. The culture medium was removed and replaced by 100 μl of 10% WST-1 in culture medium. After 1h incubation at 37°C, the A450 was measured on a Bio-Rad model 550-microplate reader. WST-1 conversion was expressed as a percentage of conversion by uninfected, untreated control cells, after subtraction of the background values of WST-1 incubated in the absence of cells.

To determine treatment interactions, the values of fraction of cell growth affected by drug dosage were subjected to the multiple drug effect analysis of Chou and Talalay [24]. Using the Calcusyn software, the combination index (CI) was determined [25]. A CI value below 0.9, between 0.9 and 1.1 or above 1.1, indicates synergism, additive effects and antagonistic effects respectively.

## RESULTS

***AdΔ24-p53 has more oncolytic potency than AdΔ24 against colon carcinoma cell lines***

To compare the anti-tumor efficacies of AdΔ24 and AdΔ24-p53 on colon carcinoma cell lines, cytotoxicity experiments were performed in which SW1398, Colo205 or WiDr cells were infected with a dose range of virus (MOI 0.03 to 100). Seven days later, cell viability was determined by WST-1 analysis (Figure 1). In all three colon carcinoma cell lines, AdΔ24-p53 caused cell death at lower viral dose than AdΔ24, indicating that AdΔ24-p53 replicated faster in these cells than the control virus lacking *p53*. Approximate IC50 values were used to compare the potencies of the two CRAds. On SW1398 and Colo205 cells, the oncolysis enhancement by *p53* expression was approximately 20-fold, whereas on WiDr cells this was approximately 4-

fold. Thus, AdΔ24-p53 was more potent than AdΔ24 in killing colon carcinoma cell lines *in vitro*.

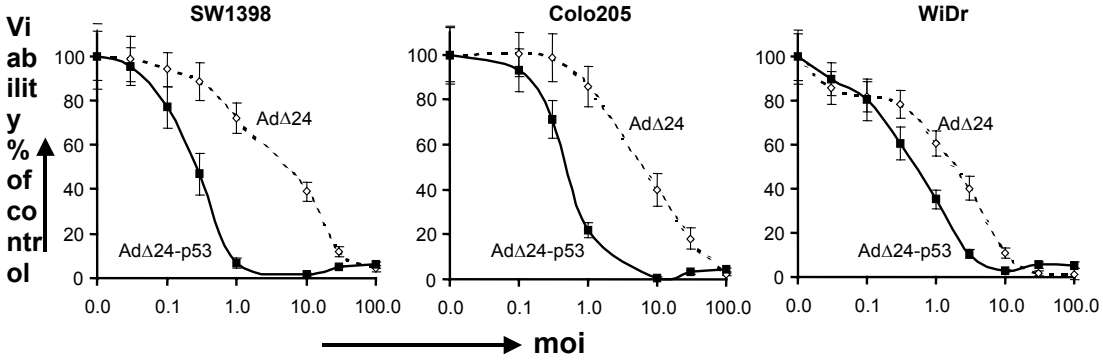
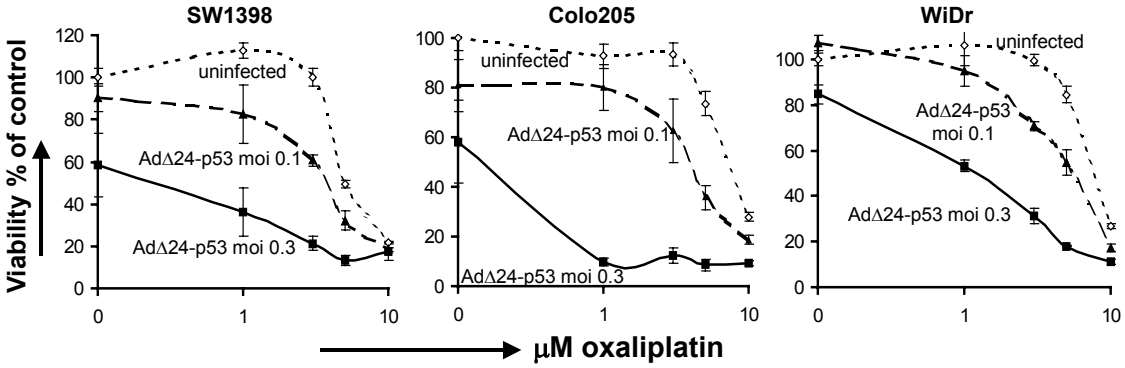



***Figure 1:*** *AdΔ24-p53 exhibits enhanced oncolytic potency compared to AdΔ24 on colon carcinoma cells. SW1398, Colo205 or WiDr cells were infected with AdΔ24 or AdΔ24-p53 at the indicated multiplicity of infection. Cell survival was determined by WST-1 conversion assay after 6 days. In all colon carcinoma cell lines AdΔ24-p53 showed greater oncolytic potency achieving comparable effects at an up to 40 fold lower viral dose.*

### *AdΔ24-p53 and oxaliplatin show synergistic cytotoxicity against colon cancer cells*

To study the combined effects of AdΔ24-p53 with the anti-cancer drug oxaliplatin, colon cancer cells were infected with AdΔ24-p53 at a low MOI and treated with different concentrations of oxaliplatin. After 6 days, cell viability of the cells was determined by WST-1 analysis (Figure 2A). Treatment of AdΔ24-p53 infected cells with a low dose oxaliplatin increased the efficacy of virotherapy against all three cell lines. The toxicity observed after combination treatment was larger than the sum of the toxicities of the two separate treatments. In order to quantitatively evaluate the effect of combining AdΔ24-p53 and oxaliplatin, the CI values were calculated using Calcusyn analysis at 50%, 75% and 90% cell viability. The average CI values for SW1398, Colo205 and WiDr were 0.8, 0.7 and 0.6, respectively. These values indicate that the combination of AdΔ24-p53 and oxaliplatin is synergistic in all three cell lines.

***2A***



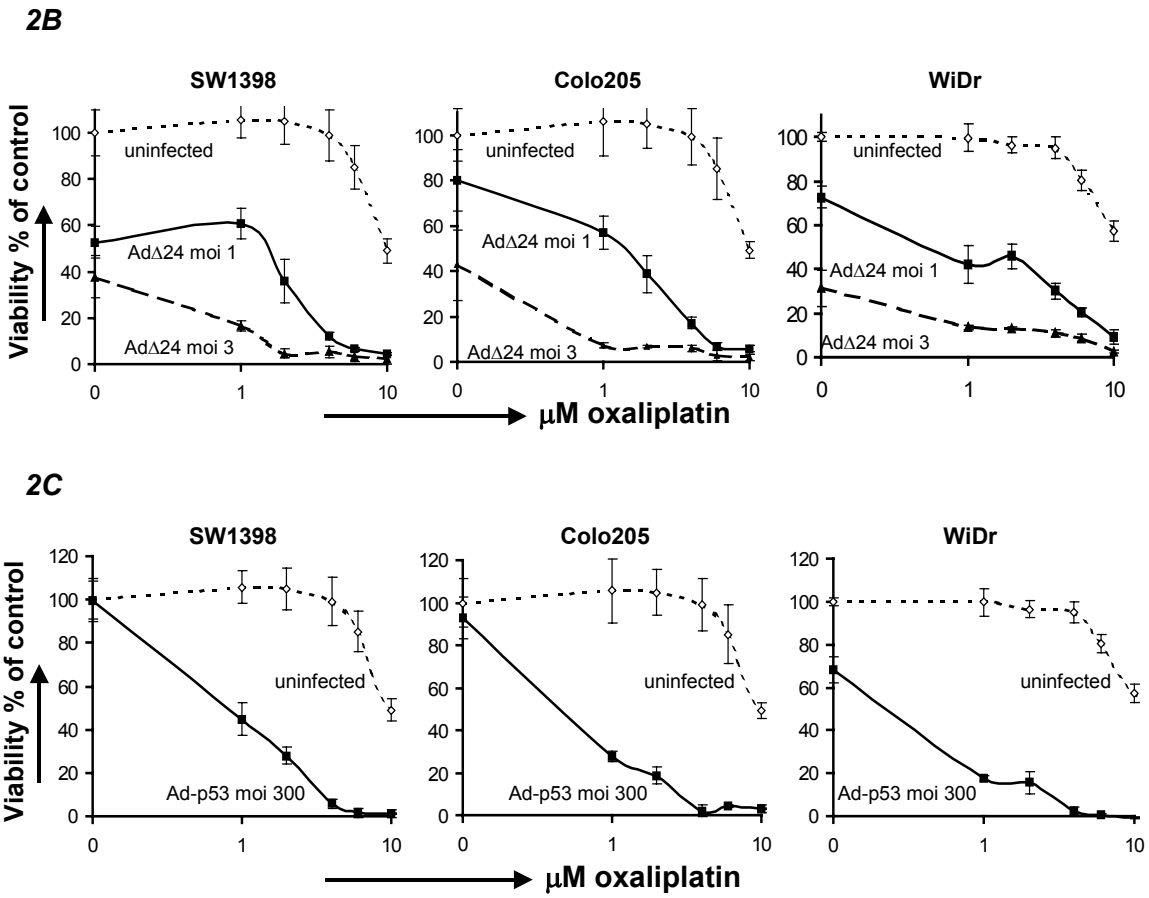



***Figure 2:*** *The efficacy of Ad∆24-p53 is enhanced by treatment with low dose oxaliplatin. SW1398, Colo205 or WiDr cells were infected with Ad∆24-p53 (A), Ad∆24 (B) or Adwtp53 (C) at the indicated MOI and subsequently treated with oxaliplatin at the indicated dose. Cell survival was determined by WST-1 conversion assay after 6 days. In all cell lines, a non-toxic concentration of oxaliplatin increased the efficacy of CRAd virotherapy or p53 gene therapy.*

### *Expression of p53 and Ad∆24 replication each sensitize colon cancer cells to oxaliplatin*

To investigate which component of AdΔ24-p53, i.e., oncolytic replication of the CRAd AdΔ24 or *p53* transgene expression, synergized with oxaliplatin treatment, these were analyzed separately. To this end, the cells were infected with AdΔ24 or with Adwtp53, a replication deficient vector expressing *p53*, and treated with oxaliplatin as described above. As AdΔ24 is less effective than AdΔ24-p53 and as Adwtp53 does not replicate, they were used at 10 and 100-times higher dose, respectively. As can be seen in figures 2B and 2C, both viruses synergized with oxaliplatin. For the combination of AdΔ24 with oxaliplatin, CI values of 0.7, 0.7 and 0.5 were obtained for SW1398, Colo205 and WiDr respectively, all indicating synergy. Altogether, these observations suggest that the three treatment components AdΔ24 replication, p53 expression and oxaliplatin each strengthened the effect of both other components.

## DISCUSSION

Oxaliplatin is a new drug for the treatment of colorectal cancer. Despite increased efficacy of oxaliplatin compared to conventional cytotoxic drugs for the treatment of colorectal cancer, there is still a need for more effective treatment modalities. Gene therapy approaches with CRAds in clinical trials that administration of these viruses to patients is a safe procedure without manifestation of severe side effects [26-30]. Unfortunately, no objective responses were documented with single agent therapy. Given this degree of safety but inadequate efficacy, second generation viruses with greater potency had to be developed. To this end, CRAds were armed with various transgenes [31-33]. Among these, expression of *p53* was found to drastically augment the oncolytic potency of the CRAd AdΔ24 in preclinical investigations [18].

In this study, we confirmed that the *p53*-expressing derivative of AdΔ24, AdΔ24-p53, was more oncolytic than its parent in colon cancer cells. We also studied combination treatment consisting of oxaliplatin and AdΔ24-p53. In all colon cancer cell lines tested, a non-toxic dose of oxaliplatin enhanced the efficacy of AdΔ24-p53 virotherapy. All three components of the combination treatment, i.e., the CRAd, the transgene and the chemotherapeutic drug were found to contribute to the efficacy of the treatment. This agrees with previous reports demonstrating that p53 sensitizes colon cancer cells to chemotherapeutic agents [34,35], including cisplatin, and that CRAds synergies with chemotherapy *in vitro*, *in vivo* and in clinical trials [21,22,36,37]. A theoretical explanation for the synergy between oxaliplatin and CRAd replication could be that chemotherapeutic drugs accelerate viral replication or outburst. We observed, however, that the number of adenoviral genomes decreased when AdΔ24 was combined with oxaliplatin treatment, compared to cells treated with AdΔ24 only (data not shown). Another potential mechanism for the synergy could be that AdΔ24 augments the antitumor activity of chemotherapy. The adenovirus E1A protein is a multifunctional transcription factor believed to be a potent inducer of chemosensitivity through both p53-dependent and independent mechanisms [38]. Malignant tumors expressing *E1A* are very sensitive to treatment with DNA damaging agents *in vivo* [39-41] and this could be an explanation for the observed synergy between oxaliplatin and AdΔ24-p53.

In conclusion, we have demonstrated that in colon cancer cell lines AdΔ24-p53 is more oncolytic than the parental control virus AdΔ24. The efficacy of AdΔ24-p53 could be further enhanced by combining it with low-dose oxaliplatin. Hence, combination therapy consisting of AdΔ24-p53 virotherapy and oxaliplatin chemotherapy might have applicability for the treatment of colon cancer. In future experiments, we would like to test combination therapy consisting of AdΔ24-p53 and oxaliplatin in subcutaneous colon cancer xenografts in nude mice.

## ACKNOWLEDGEMENTS

Dinja Oosterhoff is supported by an Educational Grant from Aventis and Victor van Beusechem is supported by a fellowship from the Royal Netherlands Academy of Arts and Sciences.

## REFERENCES

1 Ballantyne GH, Quin J. Surgical treatment of liver metastases in patients with colorectal cancer. *Cancer 1993*; **71:** 4252-4266.
2 Cvitkovic E. A historical perspective on oxaliplatin: rethinking the role of platinum compounds and learning from near misses. *Semin Oncol 1998*; **25:** 1-3.
3 Raymond E, Chaney SG, Taamma A, Cvitkovic E. Oxaliplatin: a review of preclinical and clinical studies. *Ann Oncol 1998*; **9:** 1053-1071.
4 Raymond E, Faivre S, Woynarowski JM, Chaney SG. Oxaliplatin: mechanism of action and antineoplastic activity. *Semin Oncol 1998*; **25:** 4-12.
5 Soulie P, Raymond E, Brienza S, Cvitkovic E. [Oxaliplatin: the first DACH platinum in clinical practice]. *Bull Cancer 1997*; **84:** 665-673.
6 Cvitkovic E. Ongoing and unsaid on oxaliplatin: the hope. *Br J Cancer 1998*; **77 Suppl 4:** 8-11.
7 Llory JF, Soulie P, Cvitkovic E, Misset JL. Feasibility of high-dose platinum delivery with combined carboplatin and oxaliplatin. *J Natl Cancer Inst 1994*; **86:** 1098-1099.
8 Soulie P *et al.* Oxaliplatin/cisplatin (L-OHP/CDDP) combination in heavily pretreated ovarian cancer. *Eur J Cancer 1997*; **33:** 1400-1406.
9 Scheeff ED, Briggs JM, Howell SB. Molecular modeling of the intrastrand guanine-guanine DNA adducts produced by cisplatin and oxaliplatin. *Mol Pharmacol 1999*; **56:** 633-643.
10 Page JD, Husain I, Sancar A, Chaney SG. Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair, and lethality. *Biochemistry 1990*; **29:** 1016-1024.
11 Boudny V *et al.* Biophysical analysis of DNA modified by 1,2-diaminocyclohexane platinum(II) complexes. *Nucleic Acids Res 1992*; **20:** 267-272.
12 Woynarowski JM *et al.* Oxaliplatin-induced damage of cellular DNA. *Mol Pharmacol 2000*; **58:** 920-927.
13 Oosterhoff D, van Beusechem VW. Conditionally replicating adenoviruses as anticancer agents and ways to improve their efficacy. *J Exp Ther Oncol 2004*; **4:** 37-57.
14 Post DE, Khuri FR, Simons JW, Van Meir EG. Replicative oncolytic adenoviruses in multimodal cancer regimens. *Hum Gene Ther 2003*; **14:** 933-946.
15 Fueyo J *et al.* A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene 2000*; **19:** 2-12.

16 Heise C *et al.* An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat Med 2000*; **6:** 1134-1139.
17 Hall M, Peters G. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Adv Cancer Res 1996*; **68:** 67-108.
18 van Beusechem VW *et al.* Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer Res 2002*; **62:** 6165-6171.
19 Geoerger B *et al.* Oncolytic activity of p53-expressing conditionally replicative adenovirus AdDelta24-p53 against human malignant glioma. *Cancer Res 2004*; **64:** 5753-5759.
20 Geoerger B *et al.* Expression of p53, or targeting towards EGFR, enhances the oncolytic potency of conditionally replicative adenovirus against neuroblastoma. *J Gene Med 2005*.
21 You L, Yang CT, Jablons DM. ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res 2000*; **60:** 1009-1013.
22 Yu DC *et al.* Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res 2001*; **61:** 517-525.
23 Ameyar M *et al.* Adenovirus-mediated transfer of wild-type p53 gene sensitizes TNF resistant MCF7 derivatives to the cytotoxic effect of this cytokine: relationship with c-myc and Rb. *Oncogene 1999*; **18:** 5464-5472.
24 Chou T-C, Talalay P. Applications of the median-effect principle for the assessment of low-dose risk of carcinogens and for the quantitation of synergism and antagonism of chemotherapeutic agents. *In K. R. Harrap and T. A. Connors (ed.), New avenues in developmental cancer chemotherapy, Bristol-Myers Symposium series. Academic Press, New York, N.Y. 1987***:** 37-64.
25 Chou T-C. Quantification of synergism and antagonism of two of more drugs by computerized analysis. *In D.C. Rideout (ed.), Synergism and antagonism in chemotherapy, Academic Press, NY 1991***:** 223-244.
26 Ganly I *et al.* A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res 2000*; **6:** 798-806.
27 Nemunaitis J *et al.* Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. *Cancer Res 2000*; **60:** 6359-6366.
28 Nemunaitis J *et al.* Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol 2001*; **19:** 289-298.
29 Mulvihill S *et al.* Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: a phase I trial. *Gene Ther 2001*; **8:** 308-315.
30 DeWeese TL *et al.* A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally

recurrent prostate cancer following radiation therapy. *Cancer Res 2001*; **61:** 7464-7472.

31 Mi J *et al.* Induced apoptosis supports spread of adenovirus vectors in tumors. *Hum Gene Ther 2001*; **12:** 1343-1352.

32 Zhang JF *et al.* Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy. *Proc Natl Acad Sci U S A 1996*; **93:** 4513-4518.

33 Bristol JA *et al.* In vitro and in vivo activities of an oncolytic adenoviral vector designed to express GM-CSF. *Mol Ther 2003*; **7:** 755-764.

34 Gjerset RA, Mercola D. Sensitization of tumors to chemotherapy through gene therapy. *Adv Exp Med Biol 2000*; **465:** 273-291.

35 Nielsen LL *et al.* Adenovirus-mediated p53 gene therapy and paclitaxel have synergistic efficacy in models of human head and neck, ovarian, prostate, and breast cancer. *Clin Cancer Res 1998*; **4:** 835-846.

36 Heise C, Lemmon M, Kirn D. Efficacy with a replication-selective adenovirus plus cisplatin-based chemotherapy: dependence on sequencing but not p53 functional status or route of administration. *Clin Cancer Res 2000*; **6:** 4908-4914.

37 Khuri FR *et al.* a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med 2000*; **6:** 879-885.

38 Dyson N, Harlow E. Adenovirus E1A targets key regulators of cell proliferation. *Cancer Surv 1992*; **12:** 161-195.

39 Sanchez-Prieto R, Lleonart M, Ramon y Cajal S. Lack of correlation between p53 protein level and sensitivity of DNA-damaging agents in keratinocytes carrying adenovirus E1a mutants. *Oncogene 1995*; **11:** 675-682.

40 Sanchez-Prieto R *et al.* Carcinoma cell lines become sensitive to DNA-damaging agents by the expression of the adenovirus E1A gene. *Oncogene 1996*; **13:** 1083-1092.

41 Sanchez-Prieto R *et al.* In vivo antitumor effect of retrovirus-mediated gene transfer of the adenovirus E1a gene. *Cancer Gene Ther 1998*; **5:** 215-224.

# Chapter 7

# Summary and perspectives

## SUMMARY

One of the most common types of cancer is colorectal cancer, accounting for 13 percent of all cancer cases in both sexes. This means that each year about 8000 persons are diagnosed with colorectal cancer in the Netherlands. Approximately 30% of all patients with colorectal cancer have metastatic disease at the time of diagnosis, additionally, 50% of early-stage patients will eventually develop metastatic or advanced disease. Most frequently, metastases of colon cancer are found in the liver. The initial treatment of colorectal cancer depends on the location of the tumor and its severity at the time of detection. The primary therapy for patients whose tumors have not spread to the lymph nodes is surgery with the intent to completely remove the tumor and cure the patient. In patients in which the cancer is present in the lymph nodes or the liver, surgery is combined with chemotherapy. The drug most widely used for the treatment of colorectal cancer is 5-Fluorouracil (5-FU). To increase the efficacy of 5-FU many different schedules of administration have been developed and combination therapy of 5-FU and several other cytotoxic drugs has been studied. Despite these attempts, no survival advantage was established until the development of the newer cytotoxic drugs CPT-11 and oxaliplatin.

CPT-11 (Irinotecan) is a relatively new drug for the treatment of colorectal cancer. Initially it was approved as second-line treatment for metastatic colorectal cancer, but recently it has been approved for use in combination with 5-FU/Leuovorin as first-line treatment for this disease. Administration of CPT-11 to patients is, unfortunately, limited by the occurrence of side effects, mainly myelosuppression and diarrhea. Since CPT-11 is a prodrug and needs to be converted in order to be active, a possible way to increase the efficacy and decrease the side effects of chemotherapy with CPT-11 is by using GDEPT. In GDEPT with CPT-11 the gene encoding the prodrug converting-enzyme carboxylesterase-2 (CE2) is specifically expressed by tumor cells and when hereafter the prodrug CPT-11 is administered, it will specifically be activated in the tumor. In the first part of this thesis, we aimed to increase the anti-tumor effects of CPT-11 by GDEPT using replication deficient adenoviral vectors to express the prodrug converting enzyme *CE2*. Thereafter, the utility of conditionally replicating adenoviruses for the treatment of colorectal cancer combined with GDEPT or conventional chemotherapy, was explored. An overview of GDEPT and adenoviruses is given in **Chapter 1**.

*In vivo*, for the treatment of colon cancer metastases in the liver, administration of adenoviral vectors can be performed via two different routes of administration. First, it is possible to directly inject the viral vector in colon cancer nodules in the liver, and as an alternative the viral vectors can be administered systemically by injection into the portal vein. A limitation of gene therapy with adenoviral vectors that express a therapeutic protein is that the penetration capacity of replication deficient adenoviral vectors in solid tumor masses is hampered. If the adenoviral vector is directly injected in the tumor, the virus will only infect tumor cells around the needle tract. On the other side, if the viral vector is administered via the portal vein, mainly hepatocytes will be transduced. If the gene encoding the prodrug-converting enzyme is an intracellular enzyme, like wild type carboxylesterase, it will thus only be

expressed in the cells primarily infected by the adenovirus and this will not likely increase the efficacy of CPT-11 treatment to a significant level. We therefore hypothesized that incorporating a gene encoding a secreted prodrug-activating enzyme might increase the efficacy of the therapy by enlarging the bystander effect. If the enzyme is secreted by transduced cells it will be able to freely diffuse to and through a solid tumor mass. If subsequently, the prodrug is administered it will be converted into the active drug throughout the whole tumor mass thereby exerting toxicity to untransduced tumor cells. The disadvantage of this approach is that theoretically the secreted enzyme might be able to diffuse from the tumor to the rest of the body, resulting in prodrug activation at other sites than the tumor and thus in unwanted side effects. To prevent these side effects, a secreted, tumor-targeted prodrug-activating enzyme could be used by constructing a fusion protein consisting of the secreted enzyme fused to a single chain antibody directed to a tumor antigen.

In **Chapter 2**, we describe the construction and characterization of plasmids containing the cDNA encoding a secreted form of human liver CE2 as well as a fully human fusion protein consisting of a single chain antibody against the Epithelial Cell Adhesion Molecule (EpCAM) and the secreted form of CE2. EpCAM is a highly suitable target antigen, because it is expressed at high levels on cells of many solid tumor types including colon cancer and tumor selective binding of monoclonal antibodies directed to EpCAM has been demonstrated in clinical studies. The secreted and the secreted-targeted protein were detected in the supernatant of transfected COS-7 cells and exhibited comparable enzymatic activities as determined by conversion of pNpAc, a substrate for esterases. Comparing these secreted proteins to intracellular wild type CE2, it was observed that the total esterase activity detected in the transfected cells plus the activity detected in the supernatant was much greater in cells transfected with the secreted proteins than in cells transfected with the wild type enzyme. Whether this was due to a greater amount of protein or a higher activity of the secreted enzymes is not clear. The targeted fusion protein was furthermore capable of specific binding to *EpCAM* expressing cells. Most importantly, both the secreted and the targeted form of CE2 were capable of activating CPT-11 resulting in toxicity to untransduced cells, which is very important for future *in vivo* applications.

We anticipated that both constructs could have potential for adenoviral suicide gene therapy. Therefore, we constructed replication deficient adenoviral vectors expressing these enzymes and examined their utility. In **Chapter 3,** we describe the construction and characterization of the replication deficient adenoviral vector containing the cDNA encoding secreted EpCAM-targeted CE2 and we explore the utility of this vector in a spheroid model. Spheroids are small nodules of tumor cells growing in a 3-dimensional manner and they are of intermediate complexity between *in vivo* tumors and monolayers cell cultures. Diffusion of the fusion protein from transduced cells and binding to untransduced neighboring cells was demonstrated in the multicellular spheroid model. Furthermore, treatment of colon cancer spheroids with this adenovirus, Ad.C28-sCE2, and the prodrug CPT-11 resulted in growth inhibition comparable to treatment with the active drug SN-38.

In **Chapter 4,** we describe the construction and characterization of a replication deficient adenoviral vector containing the cDNA encoding the secreted form of human CE2, Ad-sCE2, and we evaluated the utility of this adenoviral vector for the treatment of osteosarcoma, the most common primary bone tumor in children and young adults. Ad-sCE2 transduction of osteosarcoma cell lines or primary cell cultures that were brought directly into culture after the tumor was surgically removed, sensitized the cells to CPT-11 treatment, despite the fact that osteosarcoma hardly express the adenoviral receptor CAR. This suggests a very efficient bystander effect, resulting in toxicity to untransduced cells. Growth inhibition of established osteosarcoma xenografts was more effective when the tumors were injected with the adenovirus vector combined with CPT-11 treatment compared to CPT-11 or virus treatment only.

Another way to overcome the problem of limited penetration of adenoviral vectors in solid tumors is using conditionally replicating adenoviruses (CRAds). CRAds replicate only in cancer cells and destroy these cells through the natural process of adenoviral replication. Via multiple cycles of lytic replication and lateral spread, more pronounced anti-tumor effect is accomplished. Unfortunately, the efficacy of CRAds as monotherapeutic agents in clinical trials has so far been limited. The anti-cancer efficacy of virotherapy could, however, be synergistically improved by combination therapy with conventional chemotherapeutic drugs. This observed synergy warranted investigations into combination modalities consisting of virotherapy and the tumor specific GDEPT. In **Chapter 5,** we therefore investigated whether the CRAd Ad$\Delta$24, a CRAd that selectively replicates in cells with a dysfunctional pRb pathway, is compatible with carboxylesterase/CPT-11 therapy. With an *in vitro* adenovirus mixing model it was demonstrated that, in contrast to another enzyme prodrug system (thymidine kinase/ganciclovir), secreted CE2/CPT-11 treatment increased the efficacy of Ad$\Delta$24 virotherapy. Also in a more potent Ad$\Delta$24 variant, in which the adenoviral *E3* region was retained, the efficacy of virotherapy was ameliorated by secreted CE2/CPT-11 enzyme prodrug therapy. Therefore, we constructed Ad5-$\Delta$24.E3-sCE2, a derivative of the Ad5-$\Delta$24.E3 CRAd expressing the secreted form of *CE2*. This CRAd expressed high levels of functional *CE2* in human colon cancer cells. Most importantly, compared to its parental control Ad5-$\Delta$24.E3, Ad5-$\Delta$24.E3-sCE2 exhibited enhanced cytotoxicity if combined with non-toxic CPT-11 treatment on all colon cancer cell lines tested, although in the presence of CPT-11, viral replication of Ad5-$\Delta$24.E3-sCE2 was hampered. This new CRAd, therefore, has potency for the treatment of patients with colorectal cancer if combined with conventional CPT-11 treatment.

CRAds can thus be developed into more powerful agents by inserting therapeutic genes into their genome. A critical step determining the rate of virus replication is the release of newly formed virus from an infected cell through the induction of lysis at late stages of infection. A way to enhance the lysis is by expressing a pro-apoptotic gene, like *p53*. Previously, a derivative of the CRAd Ad$\Delta$24, Ad$\Delta$24-p53 was constructed that contains a constitutive *p53* expression cassette. On the majority of tested cancer cell lines, Ad$\Delta$24-p53 exhibited enhanced oncolytic potency compared to Ad$\Delta$24 [1]. In **Chapter**

**6,** we investigated whether the efficacy of AdΔ24-p53 could be further improved by combination treatment with the promising chemotherapeutic drug oxaliplatin that was recently approved for the treatment of colorectal cancer. In this study we confirmed that the *p53*-expressing variant was more oncolytic than the parental CRAd on colon cancer cell lines. An even higher anti-tumor effect was observed *in vitro* if AdΔ24-p53 virotherapy was combined with administration of non-toxic concentrations of oxaliplatin. Similar synergistic effects were demonstrated following combination treatment of oxaliplatin with AdΔ24 or with the replication deficient adenoviral vector Adwtp53. This suggested that the synergy between AdΔ24-p53 and oxaliplatin is most likely the result of two complementing mechanisms, i.e., chemosensitization by p53 gene therapy and by CRAd replication. However, oxaliplatin did not accelerate CRAd replication or viral outburst, so the actual mechanism of the observed synergy between CRAds and oxaliplatin remains unclear. In conclusion, the findings described in this chapter suggest that combination therapy consisting of AdΔ24-p53 virotherapy and oxaliplatin chemotherapy might have applicability for the treatment of colon cancer.

The following overall conclusions can be drawn from the work described in this thesis. Secreted or targeted forms of human liver CE2 can activate the prodrug CPT-11. Suicide gene therapy using replication deficient adenoviral vectors expressing these enzymes combined with CPT-11 treatment caused profound anticancer effects in *in vitro* and *in vivo* models for colorectal carcinoma and osteosarcoma. The efficacy of this approach could be further improved by combining carboxylesterase/CPT-11 enzyme prodrug therapy with the conditionally replicating adenovirus Ad5-Δ24.E3. Finally, combination therapy consisting of AdΔ24-p53 virotherapy and oxaliplatin administration is more effective in inhibiting colon cancer growth than single modality treatment.

## DISCUSSION AND FUTURE PROSPECTS

Colorectal cancer is the second leading cause of cancer-related deaths in the Netherlands, following breast cancer. The most common treatment for colorectal cancer is surgically removing the tumor. In order to also treat distant metastases, other kinds of therapies, such as chemotherapy and radiation therapy, are used. Relatively new treatment modalities for these tumors include immunotherapy, in which the body's immune system is used to fight cancer. Recent approved immunotherapeutic drugs for the treatment of colorectal cancer are Avastin (bevacizumab) and Erbitux (cetuximab). Avastatin is a recombinant humanized antibody to Vascular Endothelial Growth Factor and is approved for combination treatment with intravenous 5-FU-based chemotherapy as a treatment for patients with first-line - or previously untreated - metastatic cancer of the colon or rectum. Erbitux is a chimeric antibody directed to the Epidermal Growth Factor Receptor and is approved for combination treatment with CPT-11. Another interesting new therapeutic modality for the treatment of cancer is the use of gene therapy applications with viral vectors.

Human gene therapy is one of the new therapeutic approaches emerging from molecular biology and biotechnology revolution. The aim of human gene therapy is to correct genetic defects or to express gene products that are therapeutically useful. The principle of gene therapy for genetic diseases has several advantages over existing therapeutic modalities. These include correction of the genetic cause of the disease, selective treatment of affected cells and tissues and long-term treatment after single application. Based on these theoretical principles, at the time of its first introduction a decade ago, gene therapy promised to be an effective and safe treatment modality, which would soon cure diseases and replace classical therapies. Due to several factors it is fair to state that until today, the progress of gene therapy has been slower than expected. First of all, gene therapy is a pioneering new therapeutic modality based on complex biological systems and the incomplete knowledge of the biology of the disease and the used vector limit the effectiveness of clinical gene therapy. Secondly, stringent and time-consuming safety studies are needed as well as the establishment of new regulatory frameworks essential to ensure safety to the patient and the population. Furthermore, high costs are involved in the production of clinically approved batches of the gene transfer vectors. Finally, if successful, gene therapy will be first introduced as part of a combination therapy with other, existing therapeutic modalities, making it difficult to prove the efficacy of gene therapy.

In the last couple of years, the development of human gene therapy has further been hampered by a lot of negative publicity due to the occurrence of two tragedies. In 1999, Jesse Gelsinger died in a phase I gene therapy clinical trial and his death could be directly attributed to the vector used – an adenoviral vector. Jesse suffered from deficiency of ornithine transcarbamylase, a metabolic enzyme required to break down ammonia. The phase I trial consisted of a study in which several patients were given escalating doses of second-generation adenoviral vectors. Jesse received the highest viral dose, up to 6 x $10^{13}$ viral particles [2]. Within hours of hepatic administration, he began to experience severe complications and died two

days later. Doctors later explained that, because of a previous infection, Jesse had had an acute immune response to the adenovirus. In the months following his death, FDA and NIH officials identified a host of procedural irregularities and problems that contributed to Jesse's death.

The first gene therapy success went hand in hand with the second tragedy and was reported in 2000 when a group in Paris succeeded in totally correcting children with Severe Combined Immune Deficiency (SCID), a disease that is characterized by a total lack of T lymphocytes and natural killer cells, which normally defend the body against infections [3]. The patient's blood stem cells were incubated with a retroviral vector carrying a normal γc gene and the engineered blood cells were reinfused into the patient. Unfortunately, about 30 months after treatment, 2 of the 10 patients developed T-cell leukemia and these children had to be treated with chemotherapy [4]. The FDA subsequently halted 27 gene therapy trials. Recent findings explain why these two patients developed leukemia after gene therapy treatment [5,6]. The γc gene was inserted in both patients near *LMO2*, an oncogene that is activated as a result of translocations in acute lymphoblastic leukemia, causing overexpression of the LMO2 protein. The data of Davé *et al*. demonstrate that the γc gene can act as an oncogene when under control of a retroviral promoter, meaning that insertion of the γc vector near *LMO2* represents a double hit, and that the transduced cells are only one mutation away from tumor development. This implies that the treatment modality could be further developed into a safer gene therapy protocol, for example by modifying the vector in such way that it is not likely to activate juxtaposed genes. Since patients with SCID are forced to live in tightly-controlled, sterile "bubbles" to avoid threats to their non-existent immune systems and the gene therapy treatment improved the quality of life in such a way that the 10 treated boys in France were able to live a normal life, in my opinion it is very worthwhile to continue these kinds of gene therapy trials. All together, the latest developments in gene transfer research have demonstrated that there have been serious failures and some successes. This development may well parallel that of monoclonal antibody therapy in its early stages. In 1975, the technique to generate monoclonal antibodies was greeted with enthusiasm and monoclonal antibodies were predicted to have a profound effect on the treatment of human diseases. Indeed, these antibodies quickly became of great practical use in basic science and diagnostic analysis, but the early promise of monoclonal antibodies as therapeutic agents was slow to materialize. Progress in this area was hampered by a number of technical hurdles: therapeutically relevant targets were difficult to identify, the cost of producing purified biological reagents was very high and the first generation antibodies were themselves subject to immune responses that limited the efficacy and duration of the therapeutic effect. The past decade, however, has seen several major breakthroughs in monoclonal antibody therapeutics for cancer as well as other diseases. If the expansion of gene therapy parallels the development of monoclonal antibodies into valuable treatment modalities there's still much to be expected of gene therapy.

By far the largest proportion of gene therapy trials target cancer. For cancer gene therapy the basic concept is to introduce a therapeutic gene and its encoding product should cure or slow down the progression of the disease. This approach requires a technology capable of gene transfer specifically in

the diseased cells. To date, viral vectors have been extensively studied and modified to optimize them for this approach. Non-integrating viruses, like the adenovirus, are mainly used for the treatment of cancer. The adenoviruses are a family of DNA viruses that cause benign respiratory tract infections in humans. Advantages of adenoviral vectors are that they can transduce genetic material into both dividing and non-dividing cells and it is relatively easy to generate high-titer commercial-grade recombinant vectors. An interesting approach of using gene therapy for the treatment of colorectal cancer is to increase the efficacy of conventional cytotoxic drugs by activation of the drug at the site of the tumor. This approach is known as GDEPT or suicide gene therapy.

The aim of this thesis was therefore to further improve the efficacy of chemotherapy for the treatment of colorectal cancer by combining conventional therapy with cytotoxic drugs with adenoviral gene therapy. In the first part of this thesis we tried to improve the efficacy of chemotherapy for colorectal cancer using GDEPT with replication deficient adenoviral vectors containing the cDNA encoding a prodrug-converting enzyme. An interesting drug for colorectal cancer is CPT-11, approved for first-line treatment of this disease. CPT-11 is a prodrug that needs to be activated by the enzyme carboxylesterase (CE). In humans, this occurs rather inefficiently, only about 5% of the administered prodrug is indeed converted into the toxic drug SN-38. The most efficient enzyme to activate CPT-11 known to date is rabbit CE [7,8]. The disadvantage of a non-human enzyme is, however, that it might elicit an immune response in a patient. To prevent the occurrence of an immune response, prodrug-converting enzymes of a human origin are used, thereby allowing repeated administration of the enzyme. From recent clinical trials with GDEPT, it is clear that the responses so far were relatively poor, probably due to insufficient gene transfer and limited distribution within a tumor mass leading to inefficient prodrug activation. To overcome these problems, enhancement of the bystander effect is considered to be very important. Therefore, the development of secreted and cell-surface tethered prodrug-converting enzymes might result in better anti-tumor effects [9-11]. The disadvantage of a cell surface tethered enzyme is that it will only be expressed on the membranes of transduced cells and not on untransduced neighboring cells. Secretion of prodrug converting enzymes by transduced cells may cause a better tumor distribution, but a potential risk of secreted enzymes is leakage of the enzyme into the circulation. This could be overcome by using secreted tumor-targeted forms of prodrug-converting enzymes [11].

We indeed demonstrated that the secreted form as well as the EpCAM-targeted form of CE2 is capable of activating the prodrug CPT-11, thereby exerting toxicity to untransduced cells *in vitro* on monolayers cell cultures as well as on colon cancer spheroids. These data were, however, not confirmed in *in vivo* experiments. The problem with experiments in mice to test the activation of CPT-11 by selective expression of *CE*, is that mice have very high esterase levels in their plasma and after systemic administration of the prodrug CPT-11, most of it will be directly converted in to the toxic drug by endogenous esterases. This results in a very small therapeutic window for tumor-selective activation of CPT-11. Recently, esterase deficient SCID mice were developed by the group of Phil Potter (unpublished results). In these

mice, we would like to compare the efficacy of intratumoral injections with the replication deficient adenoviral vectors expressing intracellular *CE2* with secreted *CE2* and with secreted EpCAM-targeted *CE2* in combination with intravenous treatment with CPT-11. With this experiment it is possible to truly demonstrate that GDEPT with secreted prodrug-converting enzymes does indeed lead to an enlarged bystander effect compared to intracellular prodrug-converting enzymes. Furthermore, it would then be possible to demonstrate that targeting the prodrug-converting enzyme to tumor cells prevents leakage of the prodrug-converting enzyme into the circulation and indeed leads to fewer side effects compared to secreted prodrug-converting enzymes. A pilot study with SW1398 xenografts in these esterase deficient mice demonstrated that after intratumoral injection with the adenoviral vector expressing secreted *CE2*, no esterase activity was detectable. Apparently, the SW1398 cell line grown as xenografts, is not easily transducable with adenoviruses. Possibly, grown as a xenograft, a tumor cell line shows an altered expression of the adenoviral receptor CAR. This could possibly be overcome by targeting the virus to tumor antigens, as described in Chapter 1. Another explanation for the reduced transduction efficiency *in vivo* could be that mucus secretion or the extracellular matrix in the SW1398 xenografts prevents penetration of the virus into the solid tumor mass. This could possibly be overcome by pre-administration of mucin-degrading organic agents, like ethanol or acetone, which has been demonstrated to increase the adenoviral transduction efficiency in the bladder [12]. However, we would first like to repeat the experiments in another colon cancer model, to see whether other cell lines are infectable *in vivo* by adenoviruses. In my opinion, the probability of generating a bystander effect will be highly increased using a secreted form of CE2 instead of an intracellular enzyme. The difference between secreted and secreted, targeted enzymes is more difficult to predict. In theory, it is most likely that a targeted enzyme will indeed result in a reduction of side effects compared to a secreted enzyme, but in the case of the fusion protein described in this thesis, C28-sCE2, it is not so clear. First of all, as determined by Senter *et al*., the specific activity of human liver CE2 for CPT-11 is insufficient for targeting strategies using conjugates for antigen-specific CPT-11 activation [13]. The fact that this enzyme has proven useful in gene therapy studies is most likely attributed to very high expression levels in the transduced cells. It is thus not really likely that by leakage of secreted CE2 throughout the body, prodrug activation occurs resulting in toxicity to normal cells. The specific activity of CE2 might be improved, for example by side-directed mutagenesis, to generate a CE2 variant that has a higher specific activity for CPT-11. The opposite is also possible: to develop a prodrug that is more suited for activation by CE2, although in my opinion the biggest advantage of enzyme prodrug therapy with CE2 and CPT-11 is that the prodrug is clinically approved for the treatment of cancer. Furthermore, an important point of consideration is the affinity of the targeting moiety, in our case the single chain Fv antibody directed to EpCAM, for its antigen. The influence of the affinity of an antibody on tumor uptake has been studied previously. Adams *et al*. demonstrated that tumor uptake increased proportionally with enhanced affinity [14]. In contrast, Kievit *et al*. showed that an antibody with a very high affinity is primarily retained in the perivascular regions of a tumor [15]. The lower affinity antibody resulted in more effective

tumor growth inhibition in an ovarian cancer xenografts model than the higher affinity antibody. In GDEPT, the affinity should be high enough to prevent leakage of the fusion protein into the circulation, but as low as possible to ensure distribution of the fusion protein throughout the whole tumor. It remains to be established whether the affinity of the single chain Fv antibody used in this study is ideal.

An appealing way to improve the efficacy of GDEPT is using replication competent adenoviral vectors, as explored in the second part of this thesis. By arming replication-selective viruses with prodrug converting enzymes the therapeutic effect might be increased by a combined effect on the tumor cells of oncolysis and increased production of the prodrug-converting enzyme leading to augmented prodrug activation. On the other hand, this might also lead to a decreased efficacy, because it is well possible that prodrug activation could abrogate the viral life cycle. It has already been reported for other enzyme prodrug systems that the efficacy of the therapy is only increased when combined with slowly replicating viruses [16-18]. In this thesis, it is demonstrated that CE2/CPT-11 GDEPT combined with CRAd replication led to increased cytotoxicity to colon cancer cells because of increased enzyme production and thus augmented CPT-11 activation, although prodrug activation hampered viral replication.

In the last chapter of this thesis the efficacy of combination therapy with conditionally replicating viruses and the relatively new drug for colorectal cancer, oxaliplatin, was explored. Oxaliplatin is not a prodrug but exerts a direct effect on a cell by the formation of platinum-DNA adducts, resulting in inhibition of DNA synthesis and finally to cell death. In this thesis it is demonstrated that combination therapy was more effective in inhibiting tumor growth than single treatment modalities.

In conclusion, combining new gene therapeutic approaches using replication deficient adenoviral vectors or conditionally replicating adenoviruses for the treatment of cancer with conventional chemotherapy has prospective for further development into a highly effective treatment modality for this disease. The studies described in this thesis indicate that gene therapy remains a very good candidate to receive attention as therapeutic strategy for colorectal cancer. Advances in understanding the pathobiology, continuing basic research aimed at improving the selectivity of viral vectors and therapeutic genes should ultimately lead to the development of an effective treatment strategy for cancer.

## REFERENCES

1 van Beusechem VW *et al.* Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer Res 2002*; **62:** 6165-6171.

2 Raper SE *et al.* A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency. *Hum Gene Ther 2002*; **13:** 163-175.

3 Cavazzana-Calvo M *et al.* Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science 2000*; **288:** 669-672.

4 Hacein-Bey-Abina S *et al.* A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med 2003*; **348:** 255-256.
5 Hacein-Bey-Abina S *et al.* LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science 2003*; **302:** 415-419.
6 Dave UP, Jenkins NA, Copeland NG. Gene therapy insertional mutagenesis insights. *Science 2004*; **303:** 333.
7 Potter PM *et al.* In situ subcellular localization of epitope-tagged human and rabbit carboxylesterases. *Cytometry 1998*; **32:** 223-232.
8 Wierdl M *et al.* Sensitization of human tumor cells to CPT-11 via adenoviral-mediated delivery of a rabbit liver carboxylesterase. *Cancer Res 2001*; **61:** 5078-5082.
9 Heine D, Muller R, Brusselbach S. Cell surface display of a lysosomal enzyme for extracellular gene-directed enzyme prodrug therapy. *Gene Ther 2001*; **8:** 1005-1010.
10 de Graaf M *et al.* A fully human anti-Ep-CAM scFv-beta-glucuronidase fusion protein for selective chemotherapy with a glucuronide prodrug. *Br J Cancer 2002*; **86:** 811-818.
11 de Graaf M *et al.* Pronounced antitumor efficacy by extracellular activation of a doxorubicin-glucuronide prodrug after adenoviral vector-mediated expression of a human antibody-enzyme fusion protein. *Hum Gene Ther 2004*; **15:** 229-238.
12 Ramesh N *et al.* Identification of pretreatment agents to enhance adenovirus infection of bladder epithelium. *Mol Ther 2004*; **10:** 697-705.
13 Senter PD, Beam KS, Mixan B, Wahl AF. Identification and activities of human carboxylesterases for the activation of CPT-11, a clinically approved anticancer drug. *Bioconjug Chem 2001*; **12:** 1074-1080.
14 Adams GP *et al.* High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. *Cancer Res 2001*; **61:** 4750-4755.
15 Kievit E *et al.* Comparison of monoclonal antibodies 17-1A and 323/A3: the influence of the affinity on tumour uptake and efficacy of radioimmunotherapy in human ovarian cancer xenografts. *Br J Cancer 1996*; **73:** 457-464.
16 Lambright ES *et al.* Inclusion of the herpes simplex thymidine kinase gene in a replicating adenovirus does not augment antitumor efficacy. *Gene Ther 2001*; **8:** 946-953.
17 Stubdal H *et al.* A prodrug strategy using ONYX-015-based replicating adenoviruses to deliver rabbit carboxylesterase to tumor cells for conversion of CPT-11 to SN-38. *Cancer Res 2003*; **63:** 6900-6908.
18 Wildner O, Morris JC. The role of the E1B 55 kDa gene product in oncolytic adenoviral vectors expressing herpes simplex virus-tk: assessment of antitumor efficacy and toxicity. *Cancer Res 2000*; **60:** 4167-4174.

# Chapter 8

# Samenvatting voor niet-wetenschappers

# SAMENVATTING

Kanker ontstaat wanneer een lichaamscel genetisch ontregeld raakt en daardoor ongecontroleerd gaat delen. Deze ongecontroleerde celdeling leidt tot het ontstaan van een gezwel of tumor. De meest voorkomende vorm van kanker is borstkanker gevolgd door kanker aan de dikke darm (colon) en de endeldarm (rectum), tezamen colorectale kanker genoemd. Ongeveer 13 procent van alle kankerpatiënten heeft deze laatste vorm van kanker. Vaak wordt een tumor aan de dikke darm of de endeldarm chirurgisch verwijderd. Helaas heeft ongeveer 30 procent van de patiënten met colorectale kanker op het moment van diagnose uitzaaiingen ofwel metastasen in andere organen in het lichaam. Deze uitzaaiingen zijn meestal niet gemakkelijk chirurgisch te verwijderen en derhalve worden deze 'tumoren op afstand' vaak behandeld met bestraling en/of chemotherapie. Chemotherapie houdt in dat de tumoren behandeld worden met giftige stoffen (cytostatica) die de groei van de tumoren afremmen of zelfs de tumor vernietigen. Vaak gebruikte middelen voor de behandeling van colorectale kanker zijn 5-fluorouracil en leucovorin. Recent zijn er twee nieuwe middelen voor de behandeling van colorectale kanker beschikbaar gekomen, namelijk oxaliplatin en CPT-11.

Het gebruik van chemotherapeutische middelen voor de behandeling van kanker heeft als nadeel dat ook gezonde cellen in het lichaam door deze stoffen kunnen worden aangetast; met andere woorden, ze zijn niet tumor-specifiek. Bijvoorbeeld haarverlies en misselijkheid kunnen het gevolg zijn van behandeling met chemotherapie en deze bijwerkingen beperken de toe te dienen dosis van het cytostaticum en daardoor de effectiviteit van de behandeling. Een manier om deze bijwerkingen te voorkomen is om in plaats van de giftige (toxische) stof een niet-toxische prodrug aan patiënten toe te dienen. Deze niet-toxische prodrug kan door een enzym worden omgezet naar een actief cytostaticum. Wanneer dit enzym alleen aanwezig is in de tumor, zal de prodrug specifiek geactiveerd worden in de tumor en daardoor ontstaat lokaal een hoge dosis van het actieve cytostaticum. Dit heeft tot gevolg dat de algehele bijwerkingen van de chemotherapie afnemen, terwijl de effectiviteit van de therapie toeneemt.

Een goed voorbeeld van een prodrug is CPT-11, de drug hierboven al genoemd voor de behandeling van colorectale kanker. Om actief te zijn moet CPT-11 omgezet worden in de drug SN-38 door het enzym carboxylesterase. Wanneer CPT-11 wordt toegediend aan patiënten met kanker wordt een klein percentage (ongeveer 5%) van het CPT-11 door carboxylesterase in de lever en darmen van de patiënt omgezet in de actieve drug SN-38 en dit heeft remming van de groei van de tumor tot gevolg. Het SN-38 veroorzaakt echter ook bijwerkingen, voornamelijk diarree, die de toediening van CPT-11 beperken.

Helaas blijft dus 95% van het toegediende CPT-11 in de prodrug-vorm in de patiënt aanwezig. Het hoofddoel van het hier beschreven promotie-onderzoek richt zich op het verbeteren van de effectiviteit van behandeling met CPT-11 door tumorspecifieke omzetting van de 95% van de 'ongebruikte' prodrug CPT-11. Verhoging van de hoeveelheid prodrug-omzettend enzym carboxylesterase in de tumor zou leiden tot verhoogde effectiviteit van de therapie met CPT-11. De hoeveelheid carboxylesterase in de tumor willen we vergroten door middel van gentherapie.

Een algemeen overzicht van verschillende enzym-prodrug systemen en gentherapie benaderingen wordt gegeven in **Hoofdstuk 1**. Met behulp van gentherapie wordt een stukje DNA, in dit geval het DNA dat de genetische informatie bevat voor carboxylesterase, in de tumor afgeleverd. Vervolgens gaan de tumorcellen dit stukje DNA vertalen naar een eiwit, het enzym carboxylesterase. Wanneer vervolgens de prodrug CPT-11 wordt toegediend, zal dit in de tumor omgezet worden in de drug SN-38 en zullen specifiek de tumorcellen dood gaan. Voor het afleveren van het stukje DNA in de tumor hebben we gebruik gemaakt van een aangepast (gemodificeerd) adenovirus, ofwel een niet gevaarlijk verkoudheidsvirus. Het adenovirus dringt cellen binnen via binding aan een membraan-eiwit, zodat het DNA de gastcel, de tumorcel, bereikt. In het eerste gedeelte van dit proefschrift gebruiken we replicatie-deficiënte adenovirussen. Dit betekent dat bepaalde genen, die essentieel zijn voor de vermenigvuldiging (replicatie) van het virus, uit het adenovirus gehaald zijn. Zo'n virus is dientengevolge alleen nog maar in staat om een cel te infecteren en het DNA af te leveren; het virus is dus nog slechts een vervoermiddel voor het DNA. Het probleem met de huidige generatie adenovirussen is dat maar een klein percentage van alle cellen in een tumormassa wordt bereikt, zodat slechts een klein aantal tumorcellen het CPT-11 omzettende enzym carboxylesterase gaat maken. Om de effectiviteit van adenovirale enzym-prodrug therapie met carboxylesterase en CPT-11 nog meer te verhogen kan men denken aan een vorm van carboxylesterase die uitgescheiden wordt door cellen waarin het virus met het DNA voor carboxylesterase is binnengedrongen. Een uitgescheiden (secretoire) vorm kan namelijk diffunderen door een solide tumormassa en wanneer vervolgens de niet-toxische prodrug CPT-11 wordt toegediend, zal dit door de hele tumor heen geactiveerd worden tot de drug SN-38. Dit zal niet alleen leiden tot de dood van de tumorcellen die door het adenovirus geïnfecteerd waren, maar ook kunnen de omringende, niet-geïnfecteerde tumorcellen vernietigd worden door de gegenereerde drug. Het theoretische nadeel van een secretoir eiwit is echter dat het uit de tumor zou kunnen lekken en zodoende via de bloedbaan in andere delen van het lichaam terecht kan komen. Wanneer hierna de prodrug CPT-11 toegediend wordt, zou dit weer kunnen leiden tot veel bijwerkingen omdat de prodrug in het hele lichaam geactiveerd wordt. Om dit te voorkomen kan men het secretoire carboxylesterase genetisch koppelen aan een stukje van een antilichaam dat specifiek bindt aan een bepaald celmembraan eiwit van de tumorcellen. Bij gebruik van dit construct zal het geproduceerde fusie-eiwit gesecreteerd worden door geïnfecteerde tumorcellen, waarna het fusie-eiwit door de tumor kan diffunderen en binden aan de celmembranen van nabij gelegen tumorcellen. Ook dan zal, wanneer daarna CPT-11 wordt toegediend, dit leiden tot de dood van meer tumorcellen dan alleen de geïnfecteerde cellen.

In **Hoofdstuk 2** hebben we de constructie en karakterisatie van een secretoire vorm van carboxylesterase en een fusie-eiwit bestaande uit secretoir carboxylesterase en het antilichaam tegen EpCAM dat op membranen van tumorcellen aanwezig is, beschreven. In dit hoofdstuk hebben we laten zien dat zowel secretoir carboxylesterase als het fusie-eiwit worden uitgescheiden wanneer een stukje DNA coderend voor deze eiwitten in dikke darmkanker-cellijnen werd ingebracht. Het fusie-eiwit bleek inderdaad specifiek te binden aan cellen die het molecuul EpCAM op hun

celmembranen hadden. Tenslotte bleken beide eiwitten nog steeds in staat om de prodrug CPT-11 te activeren naar de toxische stof SN-38.

Uit deze resultaten concludeerden wij dat het zin heeft om deze eiwitten te gebruiken in combinatie met CPT-11 toediening en adenovirale gentherapie. Dit onderzoek werd daarom gecontinueerd met de constructie van replicatie-deficiënte adenovirussen die of het DNA coderend voor secretoir carboxylesterase ofwel secretoir, EpCAM getarget carboxylesterase bevatten.

In **Hoofdstuk 3** wordt de constructie en de toepassing van het adenovirus met het DNA coderend voor EpCAM getarget carboxylesterase in dikke darmkanker spheroiden beschreven. Spheroiden bestaan uit tumorcellen die in het laboratorium tot 3-dimensionale klompjes zijn uitgegroeid. Dit bleek een ideaal model om de penetratie van virussen en de verspreiding van gevormde eiwitten te bestuderen. In deze spheroiden hebben we aan kunnen tonen dat het fusie-eiwit inderdaad gemaakt wordt door cellen waar het adenovirus naar binnen was gedrongen. Ook konden we laten zien dat dit fusie-eiwit na uitscheiding door de geïnfecteerde cellen kon binden aan omringende niet-geïnfecteerde cellen. Het meest belangrijke experiment liet zien dat, wanneer we zo'n tumorklompje infecteerden met het gemodificeerde adenovirus en vervolgens de prodrug CPT-11 toedienden, het hele klompje tumorcellen vernietigd werd.

In **Hoofdstuk 4** wordt de constructie beschreven van het replicatie-deficiënte adenovirus met het DNA coderend voor secretoir carboxylesterase en de toepassing van dit virus in combinatie met CPT-11 toediening voor de behandeling van osteosarcoma. Osteosarcoma is een zeldzame tumor van het bot die voornamelijk bij kinderen voorkomt. Zowel osteosarcoma-cellijnen als primair materiaal (dit wil zeggen tumoren die operatief verwijderd zijn uit patiënten en direct daarna in het laboratorium getest worden) werden gevoelig voor CPT-11 na infectie met het adenovirus met het secretoire carboxylesterase. Deze resultaten konden ook bevestigd worden in experimenten met zogenaamde 'naakte' muizen (dit zijn muizen met een verzwakt immuunsysteem) die humane osteosarcoma-tumoren hadden.

Een andere manier waarop de penetratie van een adenovirus in een solide tumormassa verbeterd zou kunnen worden is het gebruik van conditioneel replicerende adenovirussen ofwel CRAds en dit wordt beschreven in het tweede gedeelte van dit proefschrift. CRAds zijn adenovirussen die zich, door genetische aanpassingen, wel in tumorcellen, maar niet in normale, gezonde cellen kunnen vermenigvuldigen. Wanneer een CRAd een tumorcel infecteert, zal het virus in deze cel gaan repliceren. Uiteindelijk gaat de cel dood en vervolgens komen er duizenden nieuwe virusdeeltjes vrij die op hun beurt weer nieuwe tumorcellen kunnen vernietigen. Dit type adenovirus is al toegepast in klinische trials bij patiënten met verschillende soorten kanker. Het bleek dat de effectiviteit van CRAds enorm verbeterd kon worden door combinatie-behandeling met conventionele chemotherapie. Wij waren daarom zeer geïnteresseerd in de combinatie van CRAds met tumor-specifieke chemotherapie met CPT-11 en het enzym carboxylesterase.

In **Hoofdstuk 5** wordt de constructie beschreven van een CRAd die tevens het DNA bevat voor het secretoire carboxylesterase. Dikke darmkanker-cellijnen die geïnfecteerd werden met dit virus bleken hoge

hoeveelheden carboxylesterase te maken. Wanneer de cellijnen vervolgens ook behandeld werden met de prodrug CPT-11, zagen we dat dit de effectiviteit van de therapie nog meer vergrootte.

Alle bovenstaande virussen, zowel de replicatie-deficiënte als de conditioneel replicerende, zouden dus mogelijk van toepassing kunnen zijn bij de behandeling van dikke darmkanker in combinatie met toediening van de prodrug CPT-11.

De effectiviteit van CRAds bleek dus te verbeteren door er genen in te brengen die een extra therapeutisch effect hebben. Een kritische stap die de effectiviteit van virus-therapie bepaalt, is het snel vrijkomen van nieuwe virusdeeltjes. Hoe langer dit duurt, hoe lager de effectiviteit van de therapie zal zijn, omdat er snel na toediening van het virus een immuunrespons in de patiënt op gang komt, die het virus elimineert. Een manier om het vrijkomen van nieuwe virusdeeltjes te versnellen is door een gen in te brengen in de CRAd die dit proces versnelt, bijvoorbeeld het eiwit p53. Het eiwit p53, dat ook wel 'bewaker van het genoom' genoemd wordt, kan een cel zelfmoord laten plegen wanneer dit nodig is, bijvoorbeeld bij schade aan het DNA. Vrijwel alle tumorcellen hebben dit eiwit niet meer, waardoor ze constant door blijven groeien. In het laboratorium is een CRAd gemaakt die het eiwit p53 tot expressie brengt. Uit onderzoek in het laboratorium bleek dat de CRAd met het gen coderend voor p53 inderdaad effectiever is dan de CRAd zonder p53 op vele verschillende soorten kankercellijnen, waaronder dikke darmkanker cellijnen. In **Hoofdstuk 6** hebben we gekeken of de effectiviteit van de CRAd met p53 op dikke darmkanker-cellijnen nog meer te verbeteren viel door combinatie-behandeling met het nieuwe cytostaticum oxaliplatin. Dikke darmkanker-cellijnen bleken gebaat bij toevoeging van oxaliplatin aan behandeling met dit virus. Verrassend genoeg werden ook vergelijkbare effecten op cellijnen geobserveerd wanneer we de CRAd zonder p53 gebruikten en ook wanneer we het replicatie-deficiënte virus met p53 gebruikten. Dit suggereert dat de positieve effecten van behandeling met de CRAd met p53 in combinatie met oxaliplatin het resultaat zijn van 2 mechanismen, namelijk enerzijds dat dikke darmkanker-cellijnen gevoeliger worden voor oxaliplatin wanneer ze positief zijn voor het eiwit p53 en anderzijds dat oxaliplatin een effect heeft op de replicatie van het adenovirus. Ook deze combinatie zou in de toekomst gebruikt kunnen worden voor de behandeling van patiënten met kanker aan de dikke darm.

Kort samenvattend: secretoire en tumor-bindende vormen van carboxylesterase kunnen de prodrug CPT-11 activeren. Gentherapie met replicatie-deficiënte adenovirussen die het DNA coderend voor deze enzymen bevatten, resulteert in verhoogde effectiviteit bij de behandeling van osteosarcoma en dikke darmkanker wanneer deze gecombineerd wordt met CPT-11 toediening. Dit effect was te verbeteren door combinatie met conditioneel replicerende adenovirussen. Tenslotte hebben we aangetoond dat de effectiviteit van conditioneel replicerende virussen te verhogen is door deze te combineren met behandeling met oxaliplatin. In conclusie, adenovirale gentherapie gecombineerd met (selectieve) chemotherapie lijkt veelbelovend voor behandeling van kanker. Nader preklinisch onderzoek is echter noodzakelijk om deze verworven resultaten verder te ontwikkelen in een hopelijk succesvolle, meer tumor-specifieke therapie, om zodoende de levensduur en de kwaliteit van leven van kankerpatiënten te verbeteren.

# Dankwoord

# DANKWOORD

Vaak heb ik gedacht, als ik weer eens naar een leeg Word-document zat te staren (of één met blablabla) in de hoop op inspirerende wetenschappelijke teksten, dat ik nooit aan dit dankwoord zou kunnen beginnen. Ik ben dan ook zeer blij dat het er nu toch van gekomen is en ik nu eindelijk iedereen die heeft bijgedragen aan de totstandkoming van dit proefschrift daarvoor kan bedanken.

Lieve paps en mams, ik wil jullie bedanken voor alle kansen die jullie me gegeven hebben. Met alles, ja, echt **alles**, kan ik bij jullie terecht en ik ben ontzettend dankbaar voor jullie onvoorwaardelijke liefde. Jullie interesse in alle aspecten van mijn leven, waaronder dit boekje, heeft me altijd enorm gestimuleerd!

Professor Pinedo, ik wil u bedanken dat u me de kans hebt gegeven om binnen uw afdeling promotie onderzoek te verrichten. Gedurende al die jaren bent u erg betrokken geweest bij dit onderzoek en ons (2-) maandelijks overleg bracht me altijd weer een stapje verder in de goeie richting.

Mijn beide co-promotoren: Winald Gerritsen en Victor van Beusechem. Winald, na het eerste jaar is jouw betrokkenheid bij dit project aanzienlijk toegenomen. Ook al had je het vaak erg druk, meestal was er toch wel wekelijks de mogelijkheid om de lopende zaken en lijn van het onderzoek te bespreken, bedankt daarvoor. Je enthousiasme en grote verwachtingen (in het derde jaar van dit onderzoek was het aantal door jou geschatte artikels voor dit boekje, acht!!! Helaas, dat heb ik niet waar gemaakt) hebben me altijd gemotiveerd. Victor, ofwel ‘Godfather of the PhD students’ (volgens Winald op de retraite), bedankt voor alles! Ik weet zeker dat zonder jouw inbreng dit project niet zover gekomen zou zijn en ik dit stukje derhalve nog niet zou kunnen schrijven. Toen Hidde naar Groningen vertrok, heb ik het een tijdje somber ingezien, maar ik was zeer blij toen jouw betrokkenheid toenam. Altijd was je bereid tot wetenschappelijk overleg of het vliegensvlug corrigeren van door mij gemaakte manuscripten. Ook niet onbelangrijk: het was altijd erg gezellig! Bij deze personen hoort voor mij ook Hidde Haisma. Hidde, jij hebt de basis gelegd voor dit boekje. Jouw duidelijke begeleiding in mijn eerste jaar als AIO heeft mij veel geleerd en ik vind het nog steeds jammer, maar wel erg begrijpelijk, dat je naar Groningen bent gegaan.

Uiteraard wil ik de leden van de leescommissie en de promotiecommissie bedanken voor de aandacht die zij aan dit boekje hebben besteed. Epie, ik wil je daarnaast enorm bedanken voor de hulp bij alle proefdier-experimenten.

De afgelopen 5 jaar heb ik ervaren als een intensieve, zeer gezellige periode die grotendeels bepaald werd door de geweldige collega’s op en rond het gentherapie-lab. Mijn twee nimfjes, Ida en Michelle, springen daar zelfs nog bovenuit en ten eerste wil ik jullie bedanken dat jullie daar straks samen met mij op dat podium willen staan. Michelle, mede-AIO en maatje, we hebben samen ontzettend gelachen en heel veel leuke dingen (zowel op als buiten het werk) gedaan, maar ook kon ik altijd mijn problemen en frustraties (en zelfs rare dromen) bij je kwijt. Ida, van het begin tot het einde en ook nu nog kan ik altijd bij je terecht met wat ik ook maar kwijt wil. Dank voor alles! Het was altijd gezellig. Ik ben erg blij dat mijn nieuwe baan dicht bij je in de buurt is, zodat we die gezelligheid kunnen continueren.

Daniëlle, bedankt voor het delen van 5 jaar lief en leed op onze AIO kamer, het was altijd gezellig. Enne…..dankzij jou wist ik exact waar ik al mijn spullen terug kon vinden, namelijk keurig gerangschikt en gelabeld in, op of bij jouw bureau. Petra en Jeroen, collega's van het eerste uur, bedankt voor alle gezelligheid in, rond en buiten het lab. Jeroen, ik heb het al eerder gezegd, jouw humor was geweldig, het maakte zelfs het C1-corvee aangenaam (ik vergeet nooit dat ik zat te klagen over de zware laatste maanden en het (logische!) gebrek aan tijd voor nare klusjes als corvee, en ik prompt een mail kreeg met de nieuwe lijst….8xDinja). Niet te vergeten natuurlijk alle virusbatches die je voor me geproduceerd hebt! Petra, bedankt voor heel veel uurtjes gezelligheid en je vele grappige verhalen. We blijven contact houden en ik hoop dat je in Rotterdam een toptijd zult hebben. Adhiambo (of A of Adje), dankzij de Franse uitspraken dat CPT-11 zo geweldig werkt bij osteosarcoma, zijn wij gaan samen werken. Die bewering klopte niet helemaal, maar ik heb het wel ontzettend leuk gevonden. Jammer genoeg ziet het er nu toch echt naar uit dat de plannen van samen promoveren niet door gaan… Hoop dat we elkaar nog vaak zien.
Renée, van snuffel-student uitgegroeid tot collega-promovendus, bedankt voor je vele pipetteerwerk beschreven in hoofdstuk 3. Op het moment dat ik geen spheroid meer kon zien, was jij er om dit frustrerende werkje over te nemen (ja, die ervaring bij de kaas-afdeling van AH kwam nog goed van pas bij het coupes-snijden!). Het duurde toen nog 2 jaar, maar gelukkig is ook dat stuk nu eindelijk gepubliceerd. Succes met je eigen promotie. Alle andere studenten die de gezelligheid op het lab in de loop der jaren vergroot hebben, bedankt daarvoor!
Miriam, it was a pleasure working with you! Thanks to you I finally enjoy Spanish food…..(sometimes). Lots of luck in Cambridge and I'm convinced you'll defend your thesis only a few months (weeks?) later!!! Erik (blijven we 24 bespreken?) en Jan, naast een hoop lol, kon en kan ik altijd bij jullie terecht voor wetenschappelijk advies! Harm, een beetje katten kan geen kwaad en bevordert de werksfeer, toch? Tom, toen je als AIO begon (was het 2001?), beloofde je me dat je snel weer op het C1 zou komen werken, ik wacht nog steeds op je…. De neuro-groep: Clemens, Martine en Sander, bedankt voor de vele gezelligheid. En natuurlijk ook Evelien, Aafke, Bonnie, Jacques, Eveline, Razi, Mohamed en Marco!

Alle collega's van de buur-labs wil ik bedanken voor alle gezelligheid. Jurgen en Patrick, wanneer gaan we weer jeu de bièren? Tini en Ilse, ook al kwam ik jullie de laatste weken met 10 vragen per dag lastig vallen, nooit was het te veel. Monique, het was altijd lachen buiten, zelfs in de winter! Ine, Gitta, Johan, Tanja, Toon, ik zal de gezelligheid nooit vergeten. Marcel en Dennis, succes nog met de laatste loodjes.

Ook de collega's van de afdeling Geneeskundige Oncologie, verspreid over het ziekenhuis, bedankt. Chiel, het was altijd fijn om mijn AIO hart bij je te luchten. Gaan we dat ook doen als post-doc? Tanja, gen-immunotherapie lijkt me een mooie nieuwe uitdaging. Sinéad, thanks a lot for reading the summary and discussion! Ans en Joke, de maandelijkse bezoekjes waren altijd gezellig! Van de afdeling Pathologie wil ik Ron bedanken voor de hulp met het voorkantje: hij is prachtig geworden!!!

During the last year I had the opportunity to spend two months in the lab of Phil Potter at St. Jude's Children Research Hospital. Phil, thanks a lot

for everything. I really enjoyed my time over there, and the nice dinners you and Linda prepared. I hope you don't have too many sleepless nights after May…. Chris, thanks for all your help with the *in vivo* experiments. Monica (I hope one day you will like America…), Carol, thanks for making me feel at home! From all the people of the Harris lab I would specially like to thank Katja for showing me around Memphis, watching movies (did you ever see Kill Bill 2?), shopping, etc…

Ook wil ik graag een aantal mensen buiten het lab bedanken. Eefje, al vriendinnen vanaf het moment dat ik op 4-jarige leeftijd voor jouw huis zielig op de stoep zat, hopend op contact. Je bent er altijd voor me en ik hoop er ook voor jou te kunnen zijn! Tiny (vond nog in een schriftje van vroeger de volgende opmerking: toen sag ik papa en evelin en tantetieni), bedankt dat je me binnen liet, toen ik op die stoep zat en ik denk ontzettend veel aan je! Sandra, laten we vaker afspreken!!!!

Mijn schoonouders, Olga, Salvje, Ilona en Marcel wil ik bedanken voor de interesse in de voortgang van dit boekje, maar vooral voor de vele gezelligheid van de afgelopen jaren! Gwenda, Rutger, Annick en Kylian, ik kijk al weer uit naar de volgende logeerpartij!

Maarten, ik vind het echt ontzettend jammer dat je er niet bij kunt zijn, maar het is wel begrijpelijk: geniet van je jaartje Australië! En wel terugkomen!!!

Tenslotte George: bij voorbaat instrueerde je me dat dit niet te klef mocht worden…. Ik zal dus niet al te veel opschrijven, sowieso is wat jij voor mij betekent moeilijk te verwoorden…. Ik wil je ontzettend bedanken voor alle inbreng van al die afgelopen jaren, zowel op wetenschappelijk gebied als op alle andere denkbare vlakken. Alle problemen weet je te relativeren want, het zijn je eigen woorden, er zijn altijd vele mogelijkheden.

Aan alles komt een eind, zo ook aan dit dankwoord. Iedereen enorm bedankt!!

# Curriculum vitae

# Curriculum Vitae

Dinja Oosterhoff werd op 27 augustus 1975 geboren te ’s-Gravenhage. In 1993 slaagde zij voor het eindexamen Gymnasium aan het Augustinus College te Beverwijk. In 1994 begon zij met de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. Het doctoraal examen werd in april 1999 afgelegd met als specialisatie Oncologie. In het kader van het doctoraal examen werden twee onderzoeksstages gedaan. De eerste stage werd vervuld op de afdeling Pathologie (dr. D.F. Dukers en prof.dr. J.M. Middeldorp) van het VU Medisch Centrum. In deze stage heeft ze gewerkt aan de ontwikkeling van monoklonale antilichamen gericht tegen het BARF1 eiwit van het Epstein-Barr virus. Haar tweede stage werd verricht op de Ecole Normale Supérieure te Lyon, Frankrijk (dr. A. Sergeant) en in deze stage deed zij onderzoek naar de karakterisering van de bindingsplaatsen van het virale eiwit ZEBRA aan de promoter van het Na gen van het Epstein-Barr virus.

Van september 1999 tot september 2004 was zij aangesteld als Assistent in Opleiding bij de afdeling Geneeskundige Oncologie van het VU Medisch Centrum. Het alhier verrichte onderzoek staat beschreven in dit proefschrift en is uitgevoerd onder leiding van prof.dr. H.J. Haisma (tot september 2000), dr. W.R. Gerritsen, dr. V.W. van Beusechem en prof.dr. H.M. Pinedo. Ten behoeve van dit onderzoek heeft zij 2 maanden gewerkt bij de afdeling Molecular Pharmacology van het St. Jude’s Children Research Hospital te Memphis, USA, onder leiding van Dr. P.M. Potter. In 2002 ontving zij een Scholar In Training Award voor haar bijdrage aan de AACR in San Francisco. Sinds februari 2005 is ze werkzaam als postdoc bij de afdeling Geneeskundige Oncologie (Dr. T. D. De Gruijl) van het VU Medisch Centrum. Hier verricht zij onderzoek aan het genetisch targetten van adenovirussen naar dendritische cellen.

## List of publications

M. de Graaf, E. Boven, **D. Oosterhoff**, I.H. van der Meulen-Muileman, G.A. Huls, W.R. Gerritsen, H.J. Haisma and H.M. Pinedo. A fully human anti-Ep-CAM scFv-beta-glucuronidase fusion protein for selective chemotherapy with a glucuronide prodrug. Br. J. Cancer, 2002, 86:811-8

**D. Oosterhoff**, H.M. Pinedo, I.H. van der Meulen, M. de Graaf, F.A. Kruyt, V.W. van Beusechem, H.J. Haisma and W.R. Gerritsen. Secreted and tumour targeted human carboxylesterase-2 for activation of irinotecan. Br. J. Cancer 2002, 87:659-64

**D. Oosterhoff**, M.A. Witlox, V.W. van Beusechem, H.J. Haisma, G.R. Schaap, J. Bras, F.A. Kruyt, B. Molenaar, E. Boven, P.I.J.M. Wuisman, H.M. Pinedo and W.R. Gerritsen. Gene-Directed Enzyme Prodrug Therapy for Osteosarcoma: Sensitization to CPT-11 *in vitro* and *in vivo* by adenoviral delivery of a gene encoding secreted carboxylesterase-2. Mol. Can. Ther. 2003, 2:765-71

M. de Graaf, H.M. Pinedo, **D. Oosterhoff**, I.H. van der Meulen-Muileman, W. R. Gerritsen, H.J. Haisma and E. Boven. Adenoviral vector mediated expression of a targeted fusion protein for extracellular activation of a glucuronide prodrug. Human Gene Ther. 2004, 15:229-38

**D. Oosterhoff** and V.W. van Beusechem. Conditionally replicating adenoviruses as anti-cancer agents and ways to improve their efficacy. J. of Exp. Ther. and Oncol. 2004, 4:37-57

**D. Oosterhoff**, R.M. Overmeer, M. de Graaf, I.H. van der Meulen, G. Giaccone, V.W. van Beusechem, H.J. Haisma, H.M. Pinedo and W. R. Gerritsen. Adenoviral delivery of a gene encoding a secreted, EpCAM targeted form of carboxylesterase-2 sensitizes colon carcinoma spheroids to CPT-11. Br. J. Cancer, 2005, 92:882-887

**D. Oosterhoff**, H.M. Pinedo, M.A. Witlox, J.E. Carette, W.R. Gerritsen and V.W. van Beusechem. Gene-Directed Enzyme Prodrug Therapy with carboxylesterase enhances the anti-cancer efficacy of the conditionally replicating adenovirus Ad$\Delta$24. Gene Ther., advance online publication, 2005